

**REGULATION OF E2F-1 GENE EXPRESSION IN HUMAN BREAST
CANCER CELLS**

A Dissertation

by

SHARON KHETHIWE NGWENYA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Biochemistry

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Approved as to the style and content by:

Stephen Safe
(Chair of Committee)

James Sacchettini
(Member)

David Peterson
(Member)

Gregory Reinhart
(Head of Department)

Gary Kunkel
(Member)

May 2005

Major Subject: Biochemistry

ABSTRACT

Regulation of E2F-1 Gene Expression in Human Breast Cancer Cells.

(May 2005)

Sharon Khethiwe Ngwenya, B.S., Oakwood College

Chair of Advisory Committee: Dr. Stephen Safe

17 β -Estradiol induces E2F-1 gene expression in ZR-75 and MCF-7 human breast cancer cells. Analysis of the E2F-1 gene promoter in MCF-7 cells previously showed that hormone-induced transactivation required interactions between estrogen receptor α (ER α)/Sp1 bound to upstream GC-rich sites and NFYA bound to downstream CCAAT sites within the -169 to -54 promoter region. This promoter region was also E2-responsive in ER α -positive ZR-75 cells; however, further analysis of the promoter showed that cooperative ER α /Sp1/NFY interactions were not necessary for hormone-induced transactivation in ZR-75 cells. The upstream GC-rich motifs are activated independently by ER α /Sp1 in ZR-75 but not MCF-7 cells, and the downstream CCAAT sites were also E2-responsive. E2 also induced reporter gene activity in ZR-75 cells transfected with an expression plasmid containing the yeast GAL4 DNA binding domain fused to pM-NFYA and a construct containing five tandem GAL4 response elements. Subsequent studies showed that hormonal activation of pE2F-1_{jm1} and pM-NFYA are dependent on non-genomic pathways in which E2 activates cAMP/protein kinase A. Hormone-dependent regulation of E2F-1

gene expression in ZR-75 and MCF-7 involves different mechanisms, demonstrating the importance of cell context on transactivation pathways, even among ER-positive breast cancer cell lines.

TCDD inhibited ER α -mediated responses in MCF-7 and ZR-75 cells. E2-induced E2F-1 protein and mRNA levels in MCF-7 and ZR-75 cells and this response was inhibited by TCDD. Constructs containing GC-rich sites alone or in combination with the downstream NFY sites were used in transactivation studies to investigate the mechanism of inhibitory AhR-ER α crosstalk. Although TCDD inhibited E2-induced mRNA, protein and reporter gene activity, it was not possible to determine if the inhibitory response was due to limiting ER α protein levels due to proteasome degradation since proteasome inhibitors alone block hormone-dependent responses. TCDD also inhibited the cAMP/PKA pathway by inhibiting adenyl cyclase activity. In *Drosophila* SL-2 cells cotransfected with the GC-rich -169 to -54 region, ER α and Sp1 plasmids E2 induced transactivation in cells cotransfected with AhR/Arnt expression plasmids suggesting that the AhR complex suppressed ER α /Sp1 action. These results demonstrate that TCDD inhibits E2-dependent activation of both non-genomic and genomic pathways of ER-mediated E2F-1 gene expression.

DEDICATION

To my parents Andrew and Constance Ngwenya for their love and guidance, support and always being there for me. To my brother Russell and sisters Geraldine, Elaine and Darlene for their love and support as well.

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CHAPTER I

INTRODUCTION

1.1 Cancer

Cancer is the second leading cause of death in the world. Cancer mortality rates are second only to mortality rates due to heart disease. The four most common cancers are lung, female breast, prostate, and colon cancer. These four cancers represent more than half of the cancer diagnoses and deaths in the U.S. population. In 2003, the American Cancer Society predicted that over 1.3 million new cases of cancer would be diagnosed and over 500,000 people would die as a result of cancer. Although the death rates have declined since the mid-1990s, it has been estimated that one of every four deaths is due to cancer (1, 2)

Cancer is a result of an accumulation of mutations that render the cells insensitive to growth control (3). Normal cells proliferate, differentiate, and eventually undergo apoptosis in an orderly manner; however, cancer cells are deregulated and undergo uncontrolled proliferation that results in tumor formation and metastasis. The presence of multiple mutations in cancer cells indicates that development of a malignant tumor is a complex process that occurs in a stepwise manner. This process can be described following the

This dissertation follows the style and format of Cancer Research.

classical three phase paradigm of carcinogenesis: initiation, promotion, and progression (Fig. 1).

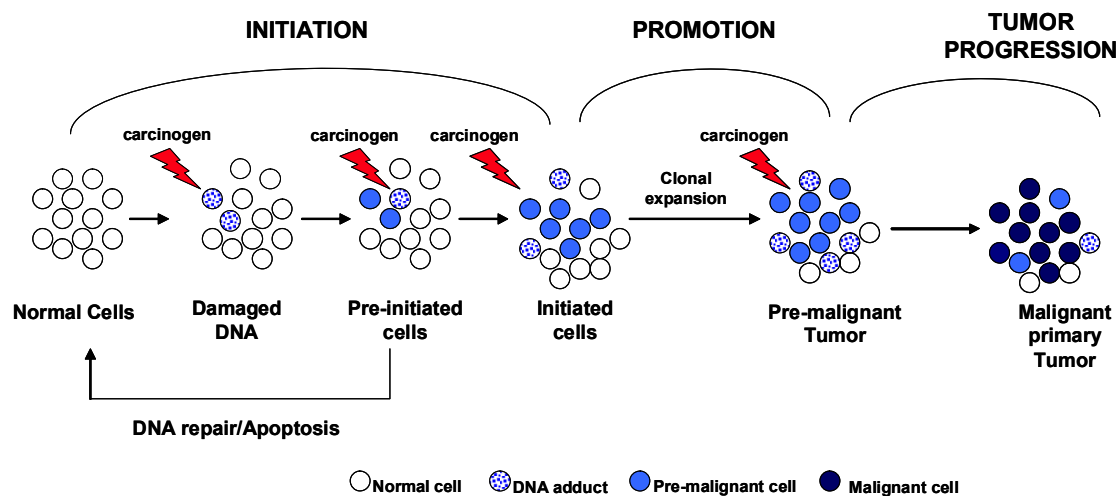


Fig. 1. Stepwise malignant progression of human cancer cells (4).

During initiation, the first phase of carcinogenesis, a genotoxic event occurs that affects the stem cells and forms premalignant lesions that have acquired one or more mutations leading to a partial escape from homeostatic control of growth (4). The genotoxic event initiates alterations in genes involved in governing either the fidelity of DNA synthesis, the efficacy of DNA repair, genes encoding proteins in the cell cycle, or regulation of apoptosis. Initiators can be biological (point mutations, loss or gain of whole chromosomes, viruses), chemical (asbestos, polycyclic hydrocarbons, nitrosamines) or physical (X-rays,

ultraviolet radiation). At this stage, the mutations are not sufficient for the development of cancer and premalignant cells must continue to grow and divide in order to form a malignant tumor.

In normal cells, DNA damage triggers checkpoint pathways that usually result in cell cycle arrest allowing the cell enough time to either repair the damage or undergo apoptosis when DNA lesions are too extensive. In at least 50% of human tumors, the checkpoint arrests are defective due to mutations in tumor suppressor genes such as *p53* and retinoblastoma (RB) which contribute to development of multiple cancers including retinoblastoma, breast and colorectal cancer (3). Therefore, as normal cells undergo apoptosis or DNA repair after genotoxic damage, cancer cells bypass these checkpoints and continue to proliferate and differentiate.

The second phase of carcinogenesis, promotion, involves clonal expansion of the initiated cell to give rise to a population of initiated cells. Promoters induce proliferation of the mutant cells but are not usually carcinogenic themselves. However, in some cases initiators can also be promoters of carcinogenesis. Promoters induce additional genetic alterations that lead to genetic instability and higher rates of chromosomal modifications. Clonal expansion increases the population of initiated cells that have acquired some of the mutations critical for carcinogenesis, thus increasing the probability that one of these cells will acquire the further genetic changes required for malignant transformation (4). Usually, an initiated cell will have no effect on the

neighboring normal cells, but clonal expansion will facilitate the tumors cell's ability to continue growing and dividing so that it can take over the environment surrounding normal cells.

The accumulation of genetic alterations during initiation and promotion subsequently leads to the conversion of the premalignant cells into malignant primary tumors. This is the third phase of carcinogenesis, progression. At this stage, the primary tumor is not invasive or metastatic. Further accumulation of mutations is required to produce new clones that are invasive and metastatic (5).

The final stage of carcinogenesis is metastasis, a series of steps in which the cancer cell leaves the original tumor site and migrates to other parts of the body through the bloodstream or lymph system (6, 7). During metastasis, the malignant cells break away from the primary tumor and attach to and degrade the proteins that surround the extracellular matrix (ECM) which separates the primary tumor from the surrounding tissue. Once the malignant cells breach the ECM, they may spread to lymph nodes near the primary tumor or send signals to the surrounding normal host tissues to initiate angiogenesis. Tumor angiogenesis promotes the formation of a new network of blood vessels that allow secondary or metastatic tumors to form in other organs and consequently starve and displace functional cells so that the organ can no longer perform its vital functions (8, 9).

1.2 Breast Cancer

Breast cancer is the most common cancer among women of all races. According to the American Cancer Society's publication *Cancer Facts & Figures 2003*, it was estimated that approximately 211,300 new cases of invasive and an additional 55,700 new cases of non-invasive breast cancer, will have been diagnosed in 2003 (2). Although breast cancer is not common in men, it was estimated that 1,300 new cases would be diagnosed in men.

Breast cancer is the second leading cause of death among women in the Western world, second to lung cancer. And in 2003, approximately 39,800 women died as a result of breast cancer (10). The reported incidence of breast cancer has risen during recent years due to the increase of mammography screening. However, the mortality rate has decreased dramatically as a result of early detection by mammography screenings and improved treatments.

1.2.1 Mammary Gland Development

The mammary gland is a structurally dynamic organ varying with age, menstrual cycle, and reproductive status. Development of the mammary organ is initiated during the embryonic phase; however, major development occurs primarily during the prepubertal phase. The mammary gland is essentially identical in both sexes during the embryonic phase. The mammary gland first appears around day 10 of embryonic development as five pairs of placodes, and by day 15 the placodes have formed epithelial buds surrounded by mesenchymal cell layers. These structures further develop into branching

network of ducts terminating in end buds through the reciprocal signaling between the epithelium, the mammary mesenchyme and the stroma fat pad.

With the approach of puberty, the mammary gland begins to show signs of growth in response to circulating hormones both in the glandular tissue and the surrounding stroma. There is an increase in the glandular size due to the growth and division of small bundles of primary and secondary ducts that eventually form club shaped structures called terminal end buds (TEB) (11). The terminal end buds give rise to new branches called alveolar buds that give rise to ducts that cluster around the terminal duct forming the lobule type 1 (Lob 1) or virginal lobule. By parturition a small ductal tree has formed (12, 13).

The normal mammary gland is comprised of two cellular compartments: the mesenchymal compartment of fatty stroma which is permeated by blood vessels and nerves and the epithelial compartment of ducts and lobules. The normal epithelial compartment is made up of three cell types, the epithelial cells which line the ducts, the alveolar cells which line alveoli and the myoepithelial cells that are between the epithelial and alveolar cells and the basement membrane. Malignant lesions of rodents and humans usually occur in the epithelial cells, specifically the undifferentiated terminal end buds, whereas benign lesions usually occur in both epithelial and myoepithelial cells (14).

During the period between birth and puberty, development of the mammary gland is quiescent. The only activity during this period would be limited ductal elongation into the mammary fat pad. The changes in the

hormonal environment during puberty are the major factors controlling mammary development. In the male, further development is inhibited by production of testosterone. In the female, estrogen acts on the mesenchymal cells to stimulate further development. The gland increases in size and the ducts extend and branch into the expanding stroma. By adulthood, a simple system of primary and secondary ducts has developed and remains in this mature state until pregnancy. With recurrent estrous cycles, some additional budding and elongation of the ducts may occur from existing buds to yield a more complex gland.

Lobule formation in the female mammary gland occurs with 1 to 2 years after the onset of menarche. The breast of an adult woman contains three types of lobules, type 1, 2 and 3. The breast of nulliparous women is mostly comprised of undifferentiated structures, such as the terminal end buds and type 1 lobules, which are sites of preneoplastic lesions which eventually progress into invasive carcinoma. Type 2 lobules are present in moderate numbers during the early years but sharply decrease after the age of 23. In parous women, type 3 lobules are the predominant structures during pregnancy and until the fourth decade of life. After 40, the number of type 3 lobules decreases. At this stage, both nulliparous and parous women's breast are similar in that they both mostly have type 1 lobules. However, it is believed that the type 1 lobules are different because in parous women the lobules are fully differentiated and are therefore less susceptible for development of malignant tumors (12, 15).

During pregnancy the mammary gland experiences the greatest and most rapid phase of proliferation. The phase is driven by the hormones 17β -estradiol (E2), progesterone (P), and prolactin (Prl). Estrogen exerts its effects mainly on the ductal system during the first trimester. Progesterone promotes alveolar development during the second and third trimester. At parturition, there is a drop in estrogen and progesterone blood levels. During this phase, there is an increase in the levels of prolactin, released from the anterior pituitary in response to a suckling stimulus from the infant. As a result, large volumes of milk are produced and lactation continues until the suckling period ends. At that time, epithelial cells are lost due to apoptosis and mammary glands are reduced in size and return to a resting phase.

1.2.2 Breast Cancer Risk Factors

1.2.2.1 Hormonal Factors

There are a number of risk factors that lead to development of breast cancer. Among the key factors are the role of inherited genetic susceptibility, environmental effects, behavioral patterns and exposure to endogenous and exogenous hormones (Fig. 2). Most of these factors affect the levels of circulating estrogens. Estrogen causes proliferation of both normal and malignant cells and this mitogenic activity contributes to the role of estrogens in initiation and promotion of carcinogenesis. The role of estrogen as an initiator is controversial. Results from the Ames *Salmonella*/microsome direct plate incorporation assay suggest that estrogen is not genotoxic and is therefore not a

mutagen (16). Furthermore, human data have failed to correlate high estrogen exposure during the time when breast cancer is most likely to be initiated, i.e. early adulthood and reproductive years, and increased breast cancer risk (17). However, other studies have reported that estrogens are able to induce direct and indirect free radical-mediated DNA damage, genetic instability, and mutations in cells in culture and *in vivo*, supporting the role of estrogen as an initiator (18). What is clear is that estrogen is a promoter that causes proliferation of malignant cells that leads to an accumulation of DNA adducts in a population of initiated cells that eventually progress to form primary tumors.

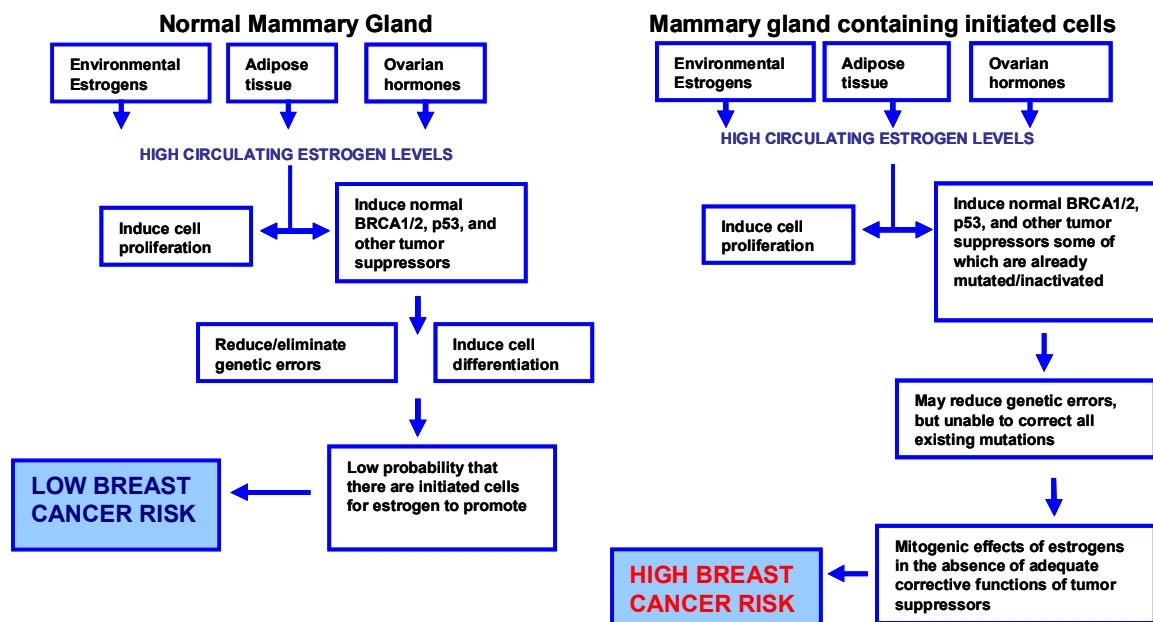


Fig. 2. The effects of circulating estrogen levels on breast cancer risk.

1.2.2.2 Inherited Genetic Risk Factors

Inherited genetic susceptibility can be attributed to about 5-10% of all breast cancer cases (19, 20). Currently, there are three known genes that when inherited in a mutated form, can confer very high lifetime risks for developing breast cancer. These genes are *p53*, BRCA1 and BRCA2.

Germ-line mutations of BRCA1 (chromosome 17q21) and BRCA2 genes (chromosome 13q12) account for approximately 40% of inherited breast cancer cases (21, 22). It is estimated that in families that have mutations in BRCA1 or BRCA2, the cumulative risk of developing breast cancer is 71% up to the age of 70, which corresponds to a 10- to 20 fold increased risk compared to women who do not have these familial gene mutations (23). Female family members who inherit altered forms of one of these three tumor suppressor genes are at high risk of developing early-onset, frequently bilateral breast cancer (Fig. 2). They are also highly susceptible to other malignancies such as ovarian cancer. The majority of inherited breast and ovarian cancer cases are the results of mutations in BRCA1, with 20 percent of the cases resulting from BRCA2 mutations (24).

BRCA1 protein is expressed in a wide variety of tissues, including the breast. Most of the expression is in tissues containing rapidly proliferating cells that are also involved in differentiation. In rodents, BRCA1 is highly expressed during embryogenesis and switches to a more tissue-specific pattern after birth. BRCA1 mRNA expression in mammary epithelial cells exhibits a cell cycle-

dependent pattern. Expression of BRCA1 mRNA was low in cells arrested in G₀ or early G₁ and at the highest during the G₁-S phase transition of the cell cycle. These data indicate that the expression pattern in BRCA1 is tightly linked to the regulation of cellular proliferation. BRCA1 is also involved in cell differentiation. Rajan *et.al* have also shown that BRCA1 mRNA levels are high in post-confluent HC11 mammary epithelial cells during differentiation (25). BRCA1 also plays an important role in repairing oxidative DNA damage. BRCA1 has been shown to interact with RAD51, a protein that is involved in DNA recombination and repair. BRCA1 also interacts with the tumor suppressor p53, supporting the idea that BRCA1 is involved in DNA repair (26).

BRCA2 has an important role in maintaining chromosomal stability through its participation in DNA recombination and repair. BRCA2 protein is expressed in the same tissues and cell types as BRCA1. Immunoprecipitation data has shown a physical interaction of BRCA2 with BRCA1 (27). Like BRCA1, BRCA2 also interacts with RAD51 and other proteins that are involved in DNA repair and recombination (28). The involvement of BRCA2 with RAD51 indicates an involvement in the repair of double stranded DNA breaks. Other studies have also shown the BRCA1 is phosphorylated as a result of DNA damage. The phosphorylation of BRCA1 is mediated by a protein kinase called ATM (ataxia-telangiectasia), which also controls the phosphorylation of other proteins involved in double strand breaks. Hyperphosphorylated BRCA1 co-localizes with BRCA2 and RAD51 proteins in nuclear foci during the S-phase of

the cell cycle. ATM is found in a nuclear complex with BRCA1 and phosphorylates BRCA1 after gamma-radiation induced DNA damage. Phosphorylated BRCA1 then activates homologous recombination with the cooperation of BRCA2, RAD51, and other DNA repair proteins (29). In contrast to BRCA1, the role of BRCA2 protein is still not very clear. Co-immunoprecipitation of BRCA2 with P/CAF, a transcription co-activator with histone acetylase activity, indicates another role for this protein as a transcription factor (30).

There are other gene variants that may impact susceptibility to breast cancer such as inherited mutations of the *p53* tumor suppressor, ataxia-telangiectasia and *PTEN* genes. *p53* has the ability to recognize, bind and repair damaged DNA, and induce cell cycle arrest and apoptosis. Germ-line and somatic mutations of the *p53* gene lead to the development of Li-Fraumeni syndrome, a rare autosomal dominant syndrome in which patients are predisposed to cancer (24, 31). This syndrome only accounts for less than 1% of the breast cancer cases.

Heterozygous carriers of mutations of the ataxia-telangiectasia (ATM) gene have been shown to be at a five-fold increased risk of breast cancer. ATM is a protein kinase that phosphorylates BRCA1, activating its DNA repair response upon DNA damage. Ataxia telangiectasia is a disease characterized by cerebral ataxia and defects of the immune system. Homozygous ATM

mutation carriers develop non-Hodgkin lymphoma at an incidence of almost 100% (32).

Mutations in the *PTEN* (phosphatase and tensin homolog) gene result in the Cowden syndrome. Carriers of mutations of this protein tyrosine phosphatase tumor suppressor gene have a risk for breast cancer and additional tumors such as follicle-cell carcinoma, thyroid and ovarian cancer, as well as polyps and cancers of the gastrointestinal tract. Female breast cancer is seen in 20-30% of the mutation carriers. However, the frequency of these inherited mutations is low among the general population, suggesting the role of other modifiable non-genetic factors such as environmental, behavioral and hormonal risk factors.

There is evidence that high levels of circulating estrogen increase the breast cancer risks in carriers of mutations in BRCA1/2, p53 and other tumor suppressor genes (17). This is due to the proliferative effect of estrogen. The mutated tumor suppressor genes allow damaged cells to escape DNA repair, apoptosis or cellular arrest, and promotion by estrogen leads to an increase in the population of initiated precancerous cells (Fig. 2).

1.2.2.3 Environmental Risk Factors

Breast cancer incidence rates in the United States are among the highest in the world and rates in Western industrialized countries are up to fivefold higher than rates in Africa and Asia (33). Additionally, second or third generation American daughters of women who migrate from the low-incidence

countries to countries of high incidence acquire the breast cancer risk prevailing in the new country (34). This suggests that environmental effects and lifestyle choices are major determinants in the development of breast cancer.

The increasing incidence of breast cancer and its geographical variations has brought greater attention to the role that the environment plays in the etiology of breast cancer. It is now believed that breast cancer is the result of a complex interaction of internally and externally introduced factors. The external factors include environmental chemicals that have been called endocrine disruptors, chemicals that interfere with the functions of the endocrine system by mimicking a hormone, blocking the effects of the hormone, or by stimulating or inhibiting the production or transport of hormones. Endocrine disruptors are mainly found in industrial settings rather than the household environments that women experience everyday. However, some of these chemicals have been found in small amounts in some households.

Organochlorines are a group of synthetic chemicals that were components of pesticides or industrial products. The most abundant of these contaminants are the pesticide dichlorodiphenyltrichloroethane (DDT) and the polychlorinated biphenyls (PCB) which were used in the US from 1945 until they were banned in 1972 (35). Dioxins are also organochlorines that were found as contaminants in herbicides. Dioxin is the general name given to 210 organic compounds containing carbon, oxygen and hydrogen with one to eight chlorine atoms. Only 17 of the 210 dioxins are known to be toxic. Dioxins can be created

naturally in trace quantities (e.g. volcanoes). They can also be created as unwanted by-products in numerous combustion processes (e.g. forest fires, cigarettes, bonfires, car engines etc.), in metal smelting and recycling processes and in the manufacture of a few chlorine-containing chemicals. These chemicals are known to have estrogenic and antiestrogenic activity, which may be important in increasing or decreasing susceptibility to breast cancer (36). Chemicals in this group may also reduce cell-mediated immune functions, which would subsequently increase susceptibility to breast cancer (37). In 1993, Wolff and colleagues investigated the role of organochlorines in breast cancer (38). They found a two to four-fold increase in the occurrence of breast cancer among women with the highest serum levels of dichlorodiphenyldichloroethylene (DDE) a metabolite of DDT, and PCBs compared to those with the lowest levels. However, since the publication of this study, there have been several reports using larger numbers of patients/controls showing levels of PCBs and DDE were not higher in breast cancer patients compared to controls ruling out the causative effect of organochlorines (39-41).

Exposure to metals has also been associated with breast cancer risk. In MCF-7 breast cancer cells, divalent cadmium, copper, cobalt and other metals activated responses mediated by the estrogen receptor α (ER α) (39). It was found that this activation was more potent than that of phytoestrogens, most environmental estrogenic chemicals of concern, and the selective estrogen receptor modulators being used to treat breast cancer. However, ongoing

studies in this laboratory suggest that metal ions may only be weakly estrogenic (42, 43). The most well established environmental risk factor is ionizing radiation. It has been shown in laboratory animals as well as humans that relatively high doses of ionizing radiation increase the rates of breast cancer susceptibility. The most convincing human evidence comes from studies on survivors of the atomic bomb blast in Hiroshima and Nagasaki who were exposed to gamma radiation (39). Women who survived the atomic blasts had a 1.4 to 2.2-fold increased risk for developing breast cancer. Furthermore, studies of adults who survived childhood cancer through radiation treatment indicated that breast cancer was the most common second malignancy regardless of gender.

1.2.2.4 Behavioral and Lifestyle Risk Factors

Although rates of breast cancer vary widely by geographic areas, only a small component of these differences is due to genetics or exposures to environmental chemicals. It is likely that differences in lifestyle choices and health behaviors constitute the major risk factors for breast cancer. Lifestyle and health factors are usually non-genetic risk factors that can be modified and these include obesity, diet, reproductive factors and exogenous use of estrogens.

Obesity and a sedentary lifestyle account for an estimated 25% of breast cancer cases worldwide. In a study conducted by the American Cancer Society, it was found that the risk of breast cancer mortality significantly increased with increasing levels of obesity (44). Some studies have linked high-fat intake with an increase in breast cancer risk while other studies show no correlations between a high-fat diet and breast cancer risk (17, 45, 46). Both human and animal studies indicate that a high-fat diet may increase the estrogen receptor content in breast tissue. A high-fat diet may also increase the amount of adipose tissue in the body. Adipose tissue produces estrogen through the aromatization of androgens to estrogens (47, 48). Estrogen produced from adipose tissue is the main source of estrogen for postmenopausal women. Overweight and obese postmenopausal women have higher levels of estrogen than their leaner counterparts (49, 50). They also have higher insulin levels, which promotes cancer cell growth. Regular exercise has been shown to reduce the concentration of insulin in the bloodstream (51). Therefore, higher circulating

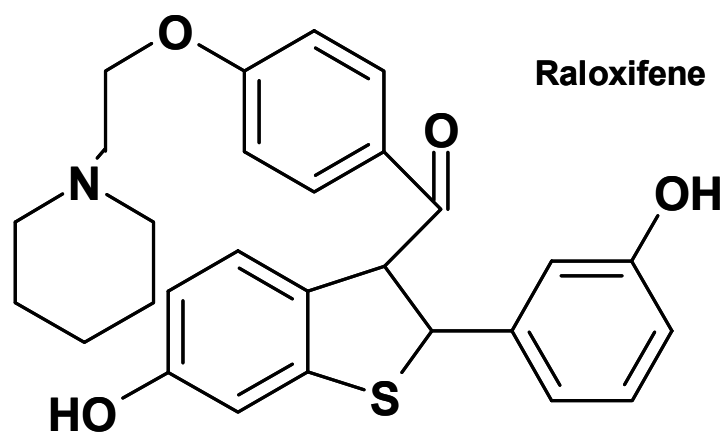
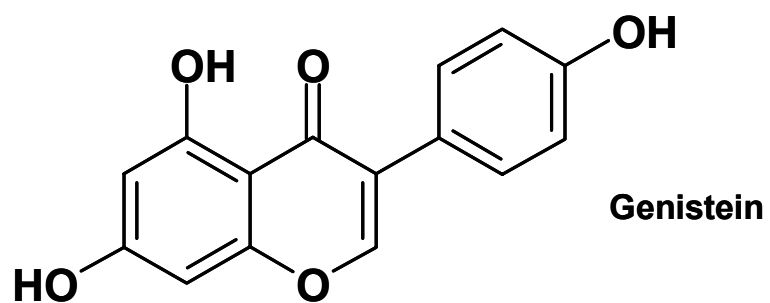
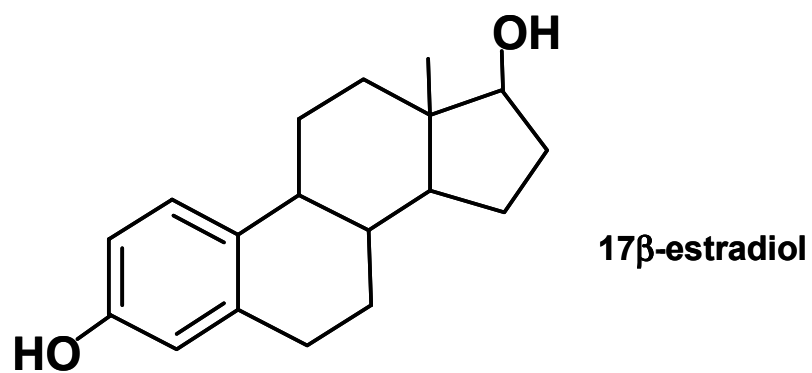


Fig. 3. Structural similarity exists between estradiol (an estrogen), raloxifene (a SERM) and genistein (a phytoestrogen).

concentrations of the hormones estrogen and insulin in overweight and obese postmenopausal women would explain the increased risk for breast cancer.

Epidemiological and experimental studies suggest that a diet rich in phytoestrogens may have beneficial effects on health (52). Phytoestrogens are non-steroidal plant-derived compounds that possess weak estrogenic effects. The chemical structure of genistein, an isoflavone derived from soy products, is similar to that of estrogen and raloxifene, a selective estrogen receptor modulator (SERM) (Fig. 3) (53). The structure similarity allows the phytoestrogens to compete for the estrogen receptor. Since phytoestrogens are generally weaker than endogenous steroidal estrogens, it has been suggested that their competition for the estrogen receptor would be antagonistic. Asian populations exhibit a decreased risk of estrogen-related diseases such as cardiovascular disease, menopausal symptoms, postmenopausal osteoporosis, and breast cancer. Protection from these conditions may be a result of a diet high in phytoestrogens due to the high consumption of soy products (52). Generally, population studies in Asian countries such as Japan and China, as well as the United States indicate that phytoestrogens have a protective effect against breast cancer and other estrogen-mediated conditions (41). However, there are some concerns that estrogenic properties of phytoestrogens, although weak, can be detrimental to women who are at high risk or have breast cancer (54, 55).

1.2.2.5 Endogenous and Exogenous Estrogens

The role of endogenous and exogenous estrogens is the strongest and most consistent risk factor associated with breast cancer. A woman's reproductive history plays a key role in determining her exposure to endogenous estrogens. Early menarche and late menopause are factors that increase the risk of breast cancer, whereas premenopausal bilateral oophorectomy before the age of 35, which eliminates ovarian estrogen exposure, decreases the lifetime risk by nearly 75% (47). Studies of the relationship between breast cancer and early age at menarche indicated that breast cancer risk is decreased by 10-20% for each additional year before the onset of menarche (56). Early menarche is associated with an earlier onset of ovulatory cycles and late menopause increases the number of cycles and results in longer exposure to endogenous estrogens, which increase cellular proliferation (47, 57). Increased proliferation of the mammary epithelium can enhance promotion of the carcinogenic process. Cancer initiation requires the interaction of a carcinogen with an undifferentiated and highly proliferating mammary epithelium. Therefore longer exposure to ovarian hormones results in increased probability for carcinogens to interact with the proliferating mammary epithelium.

Parity and the age at first full term pregnancy have been identified as protective factors against breast cancer. Although parous women have a decreased risk compared to nulliparous women, it is the age of their first delivery that is the most protective (21, 58). Some reports indicate that multiparity may

be protective, however Trichopolous and coworkers showed that the age at first birth (before 28 years) was still the most important factor (59). In studies that investigated the relationship between breast cancer and parity, each full term pregnancy led to 10-15% decrease in risk for breast cancer (56). Full term pregnancy leads to differentiation of the mammary gland resulting in the removal of a population of highly proliferating cancer-susceptible cells. Mammary gland differentiation involves the interaction of ovarian, pituitary, and placental hormones that induce inhibition of cell proliferation (60). A mammary gland that has encountered a full term pregnancy at an early age undergoes differentiation that decreases the number of cancer-susceptible cells at an early age, thereby decreasing the overall risk of breast cancer.

Since the 1960s, female sex hormones have been used as contraceptives. These hormonal contraceptives are usually composed of synthetic or natural versions of estrogens and progestins. Theoretically, the use of exogenous hormones over the course of a woman's life could increase the risk of breast cancer by increasing the opportunity for initiation, or by promoting genotoxic events occurring at an early age. However, results from studies of the effects of oral contraceptives (OC) on breast cancer risks have produced controversial results. European studies from the United Kingdom and the Netherlands have reported an increased risk associated with OC use in women diagnosed before the age of 36 (61, 62). Other studies also indicate that early use of OCs increases the risk of breast cancer. However, most of the results

vary with parity and age at diagnosis (63). Most studies have reported that there is no significant association between OC use and breast cancer risk (64, 65). In many cases, women who used OCs and were diagnosed with breast cancer had significantly fewer full term pregnancies and were older at the first term of pregnancy (66). Reproductive behavior is more likely to be the cause of the increased risk rather than use of OCs.

There is an increasing concern that the use of hormonal replacement therapy (HRT) may be a risk factor for breast cancer. HRT is the peri- and postmenopausal use of sex hormones, mainly estrogen alone or in combination with progesterone, to relieve climatic symptoms associated with menopause (67). Breast cancer is more frequently diagnosed in women who have a history of prolonged use of HRT. HRT may have a potential role in the activation of dormant cancer cells rather than induction of malignant transformation (68). Results for studies of the risk of HRT and breast cancer are controversial. While most studies indicated that women who use HRT are not at a higher risk of breast cancer than their counterparts who do not use HRT, the use of HRT is generally not recommended for women who are at high risk for breast cancer (69, 70).

1.3 Estrogen

17 β -Estradiol (E2) is the most abundant naturally occurring estrogen in the body followed by estrone (E1) and estriol (E3). Estradiol is mainly secreted by the ovaries and is the predominant estrogen during the premenopausal

period. Estrone is the main estrogen after menopause and is synthesized in the adipose tissue from adrenal dehydroepiandrosterone. Estriol is the principle estrogen formed during pregnancy, and is produced primarily in the placenta (71).

1.3.1 Estrogen Synthesis

Bioactive estrogens are synthesized from inactive steroids as well as androgenic precursors (androstenedione or testosterone) and estrone sulfates through aromatization and hydrolysis of the sulfate group respectively (Fig. 4). Aromatase (also known as *CYP19*), a member of the cytochrome p450 superfamily of genes, is the key enzyme in estrogen biosynthesis. Aromatase catalyzes the aromatization of the androgen androstenedione, an inactive androgen secreted from the adrenal cortex or gonads, to estrone (72). Over 70% of breast carcinoma specimens express aromatase activity. Most of the activity was detected in stromal cells and adipocytes adjacent to the carcinoma, indicating a correlation between aromatase expression with carcinoma invasion. Subsequently, aromatase inhibitors have been used as treatment for breast cancer (73).

1 (Fig. 4) (75). Both estradiol and estrone can be hydroxylated by 16α -hydroxylase (Fig. 4) (74).

1.3.2 Estrogen Metabolism

There are several hypotheses concerning the mechanisms whereby estrogens cause breast cancer (Fig. 5). The first and most commonly held hypothesis is that estrogens bind to the estrogen receptor and stimulate the transcription of genes involved in cell proliferation and differentiation. The second and more controversial hypothesis is that estradiol forms genotoxic metabolites that directly damage DNA. There has been some data supporting the genotoxic metabolism hypothesis (76, 77). However, most of these data are either not repeatable or provide indirect evidence and do not completely rule out the classical estrogen receptor hypothesis.

In the genotoxic metabolism hypothesis (Fig. 5), estradiol is hydrolyzed to 4-OH-estradiol by CYP1B1, a member of the cytochrome P450 superfamily. Breast cancer tissue exhibits significantly higher levels of 4-OH-estradiol and CYP1B1 than normal breast tissue (78, 79). 4-OH-Estradiol is then converted to estradiol-3,4-quinone, which can bind guanine or adenine. 4-OH-estradiol-guanine or 4-OH-estradiol-adenine is released from the DNA and leaves depurinated DNA. At this point, DNA repair occurs in normal cells, however, in

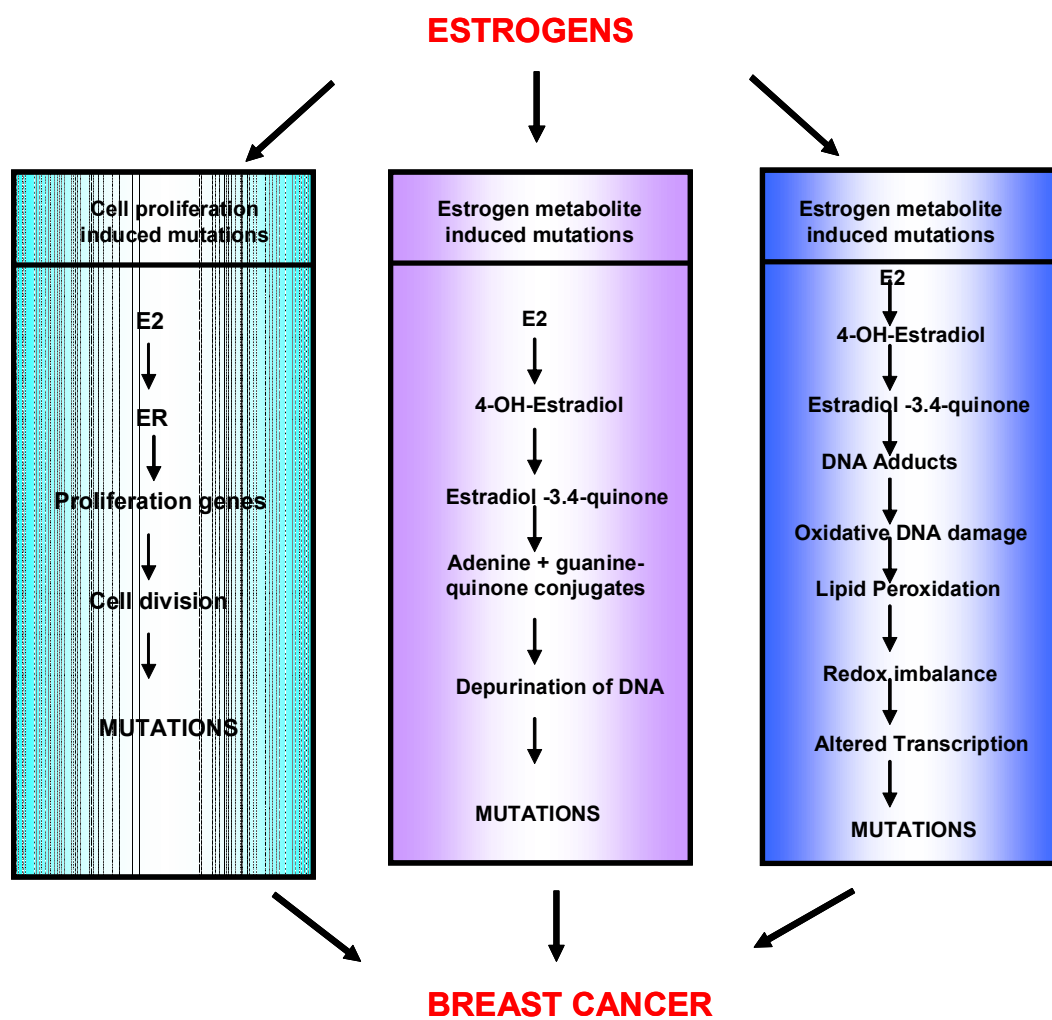


Fig. 5. Mechanisms of E2-induced mammary carcinogenesis (76, 77).

cases where DNA repair is faulty, point mutations which serve as potential initiators of neoplastic transformation are formed (80). This pathway is hypothesized to act additively or synergistically with the estrogen receptor pathway to induce breast cancer.

In another form of the genotoxic hypothesis, the quinone metabolites of estrogen can also cause oxidative damage to lipids and DNA through redox cycling processes that produce reactive oxygen species (ROS) where oxygen is partially reduced to form a free radical that forms DNA adducts with all bases of DNA instead of only the guanines (Fig. 5). If a cell has antioxidant enzymes and radical scavengers which mitigate the toxicity of the free radicals, DNA damage can be repaired. However in cases where there is excessive generation of ROS, this could lead to disruption of cellular redox homeostasis and, as a consequence, an alteration of transcription factor function, causing inappropriate alterations in the regulation of gene expression (81).

1.4 Transcription

1.4.1 Chromatin Structure

The human genome is comprised of approximately three billion base pairs of deoxyribonucleic acid (DNA) that express over 30,000 genes. DNA is contained in a compact area and is seen as a mass of chromatin in eukaryotic nuclei during interphase. The packaging of chromatin changes during the cell cycle; at the time of cell division, mitosis or meiosis, the genetic material becomes even more tightly packaged into individual chromosomes. Chromatin has a

compact organization in which most DNA sequences are structurally inaccessible and functionally inactive. Within this mass are the majority of active sequences required for transcriptional activation of specific genes.

There are several hierarchies in the organization of chromatin. The first level is the winding of DNA into the bead-like particles, forming a 10 nm fiber that is a component of euchromatin, heterochromatin, and chromosomes. At this level, a continuous duplex thread of DNA runs through a series of particles called nucleosomes. The nucleosome contains ~200 bp of DNA associated with a histone octamer that consists of two copies each of the core histones. The second level of organization is the coiling of the nucleosome into a helical array to constitute the ~30 nm fiber that is found in both interphase chromatin and mitotic chromosomes. The third level of organization is the packaging of the fiber itself into chromosomes.

In order for DNA to be synthesized or transcribed, the structure of chromatin must be unfolded in order to gain access to the coding sequences. Transcription factors bind to the nucleosome and subsequently recruit enzymatic activities which will alter the chromatin in a way that permits the recruitment of the basal transcription machinery and other factors (Fig. 6). The unfolding is made possible through acetylation of histone core proteins by histone acetyltransferase enzymes (82). Histone acetylation causes direct physical changes in the secondary structure of the nucleosome by neutralizing the

positive charge of the scaffolding proteins (83). Changes in the nucleosomes secondary structure modify protein-protein and protein-DNA interactions.

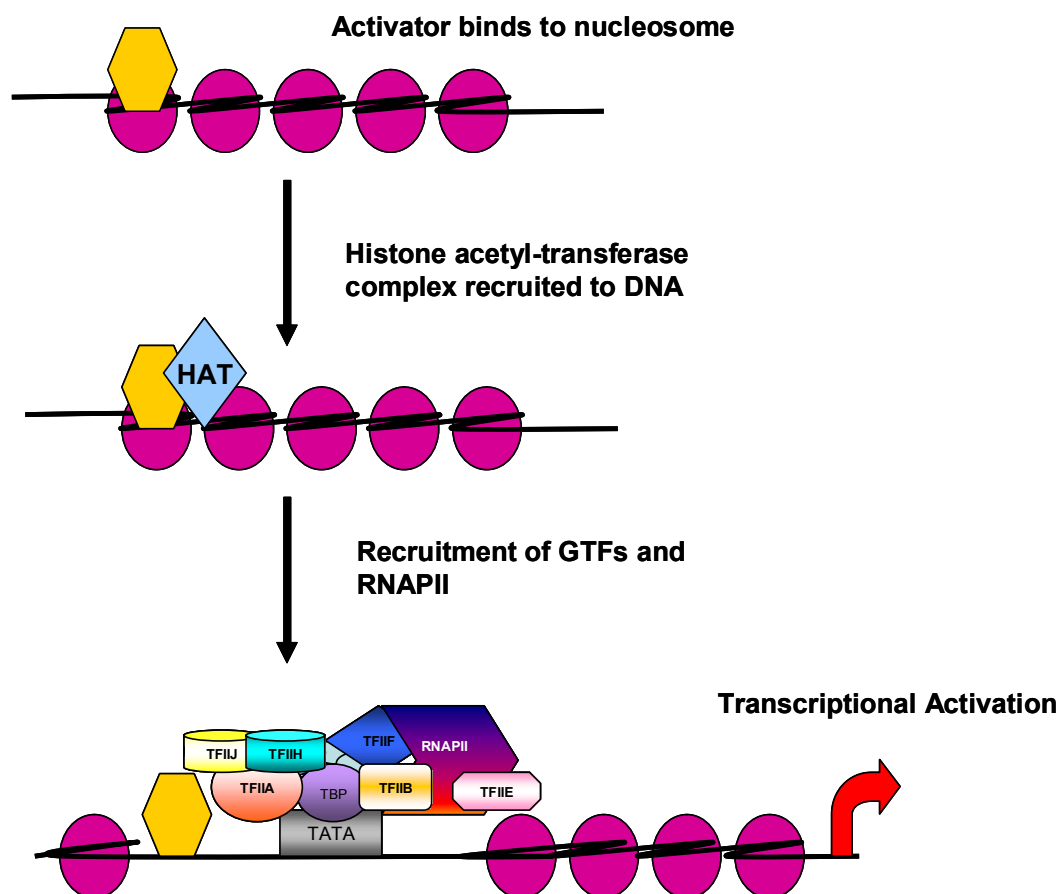


Fig. 6. Chromatin modification by histone acetyl transferase. GTF - general transcription factors. RNAPII – RNA polymerase II.

1.4.2 Transcription Initiation

Activation of genes to express their encoded proteins is dependent on both transcription and translation. Transcriptional regulation of gene expression can be divided into three distinct steps, namely, initiation, elongation, and termination. In order for RNA polymerase II (RNAP II) to transcribe a gene, it needs to be recruited to the promoter, assembled with general transcription factors (GTFs), and then initiate the transcript. DNA serves as a template for the synthesis of ribonucleic acid (RNA) much as it does for its own replication. Protein-coding genes have a number of sites that are important for gene expression. The transcription start site is where a molecule of RNA polymerase binds to begin transcription. There are three types of RNA polymerase. RNA polymerase I transcribes 28S, 18S, and 5.8S ribosomal RNA (rRNA) that is used in the building of ribosomes, the machinery for synthesizing proteins by translating messenger RNA (mRNA). RNA polymerase II (RNAP II) is found in the nucleus and is comprised of a complex of 12 different proteins. RNAP II is responsible for synthesizing mRNA from the DNA template. RNA polymerase III is located outside the nucleolus, transcribing 5S rRNA (a part of the large subunit of the ribosome), U6 snRNA, some small RNA, and all tRNA genes that carry amino acids to the growing chain of RNA (84).

Transcription is initiated by RNAP II at the proximal promoter, the region within the immediate vicinity of the transcriptional start site (85). The promoter can be divided into core and regulatory elements. The core elements are the

sites for assembly of the preinitiation complex. The core elements can include the TATA box and an initiator sequence (86). These elements may be found together or separately on gene promoters. The regulatory elements include enhancers and silencers that allow transcription to be regulated from a distance.

The TATA box is a sequence of seven bases (TATAAA) upstream of the start site on the 5' side of the gene. It has been observed that although TATAAA is the consensus sequence, variations of this sequence are also functional (85, 86). The TATA box is usually located 25 to 30 bp from the start site. During transcription, the TATA box is bound by a large complex of over 50 different protein transcription factors that form the preinitiation complex in a stepwise manner (Fig. 7) (84). At first, the general transcription factor IID (TFIID) which is a complex of TATA-binding protein (TBP) and eight other protein factors known as TBP-associated factors (TAFs) which bind to TBP and each other but not to DNA, recognizes and binds to the TATA box. TFIIA then binds TFIID to form a D-A complex that is thought to stabilize the TFIID-DNA interactions. TFIIA binding is followed by TFIIIB, the only member of this complex that binds both DNA and RNAP II. Finally, TFIIF recruits RNAP II to the promoter and preinitiation complex is formed after the addition of TFIIIE, and TFIIH. At this point, some of the factors leave the preinitiation complex, and promoter.

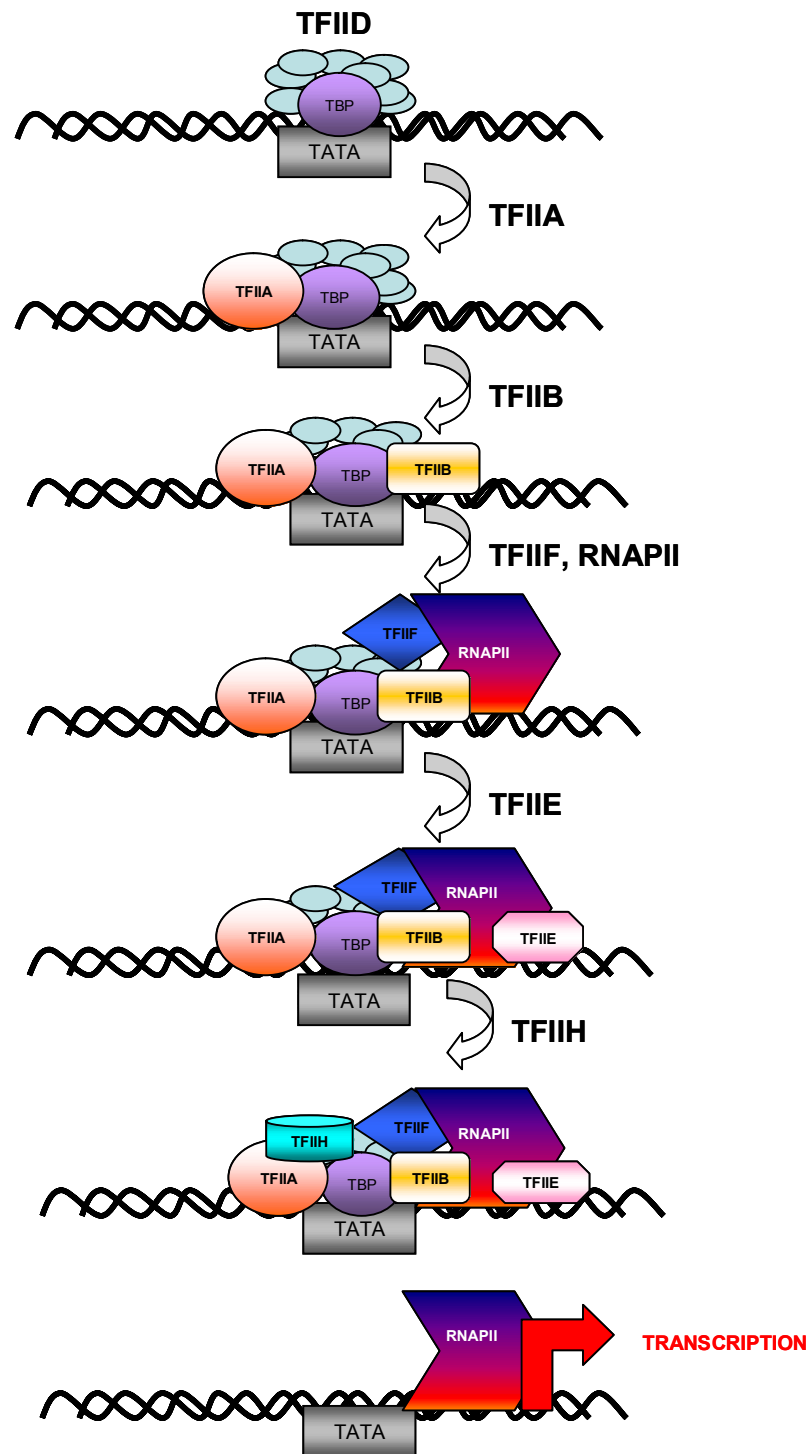


Fig 7. Formation of the preinitiation complex (82).

clearance occurs as contact between the promoter and RNAP II is broken, and RNAP II initiates transcription (87).

Initiator elements (Inr) encompass the transcriptional start site. The location of the Inr sites can be determined by spacing from the TATA box (85). However, Inr can also be found in TATA-less promoters. In the case of TATA-less promoters, Inr acts as the site for the assembly of the preinitiation complex and there is evidence that Inr is bound by TFIID in a sequence specific manner (86). More specifically, some of the TBP-associated factors (TAFs) in the TFIID complex are responsible for interaction of TFIID with Inr. RNAP II is also able to recognize and bind Inr and mediate transcription in an Inr-dependent manner. Therefore, TFIID and RNAP II can be recruited to the promoter in a TATA-dependent and TATA-independent manner.

Enhancers and silencers are *cis*-acting DNA sequences that increase and decrease transcriptional rates, respectively. Some transcription factors bind to enhancer regions of the DNA that are thousands of base pair away from the gene they control (88, 89). Enhancers can be located upstream, downstream, or even within the gene they control. Most enhancer binding proteins interact with their cognate DNA binding sites and also bind transcription factors assembled at a specific gene promoter (90). These sites cause the enhancer binding proteins to draw the DNA into a loop that allows interaction with proteins assembled at the promoter region and thereby facilitate target gene expression (Fig. 8).

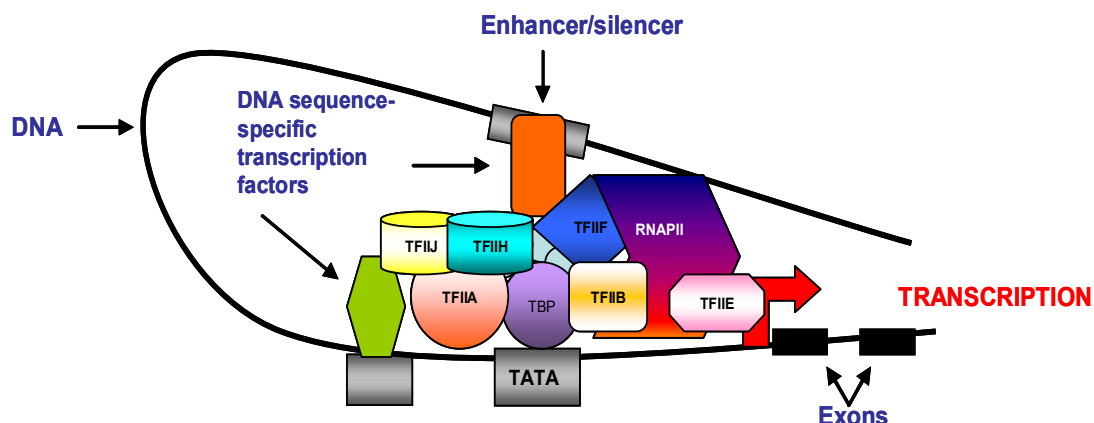


Fig. 8. DNA bound with transcription factors.

Silencers are control regions of DNA that, like enhancers, may be located thousands of base pairs away from the gene they control (85). However, when transcription factors bind to them, target gene expression is repressed.

TFIIH has DNA helicase activity that is responsible for unwinding DNA in order to allow RNAP II to transcribe DNA into mRNA (91). TFIIH is recruited to the DNA and the resulting RNAP II and transcription factor complex induces the 12-15 bases of the DNA double helix to separate forming a transcription “bubble” that allows RNAP II to proceed down one strand reading the DNA message in a 3' to 5' direction (92). In eukaryotes, this requires the removal of the nucleosomes in front of the advancing RNAP II. A complex of proteins is responsible for the removal of the nucleosome. The same complex is also responsible for replacing nucleosomes after the DNA has been transcribed and RNAP II has moved on.

As the RNA polymerase travels along the DNA strand, it assembles ribonucleotides into a strand of RNA. Each ribonucleotide is inserted into the growing RNA strand and RNA is synthesized in a 5' to 3' direction. As each nucleoside triphosphate is brought in to add to the 3' end of the growing strand, the two terminal phosphates are removed and a phosphodiester bond is formed (91). After the formation of the phosphodiester bond, two to three unstable bases are synthesized and abortively released by RNAP II. The abortion of two to three base transcripts is followed by the generation of longer transcripts of approximately 11-15 bases and the closing of the opened upstream DNA region. The downstream part of the transcription bubble can then continue to expand to allow the procession of RNAP II with the concomitant closure of the upstream region.

Transcriptional termination occurs when RNAP II encounters a termination signal on the DNA. Unlike initiation, termination does not always occur at the same bases, but at a zone downstream that contains loosely defined terminator sequences. When the terminator sequence is encountered, RNAP II and its transcript are released from the DNA and RNAP II is recycled in order to participate in another round of transcription (92).

1.4.3 Transcription Factor Sp1

Specificity protein 1 (Sp1) is a member of the Sp/KLF (Krüppel-like factor) family of proteins that have been shown to interact with GC-rich promoter elements. Dynan and Tjian first identified Sp1 in 1983 by its ability to selectively

bind and activate transcription of the viral SV40 promoter (93). Sp/KLF proteins bind to a GC-rich decanucleotide sequence known as the “GC-box” with a consensus sequence of GGGCGG. Subsequent studies revealed that multiple genes that contain GC-boxes are activated by Sp1 and related Sp proteins, and these include genes such as thymidine kinase (94, 95), insulin-like growth factor-1 receptor (96), dihydrofolate reductase (DHFR) (97), growth hormone (GH) receptor (98), and alcohol dehydrogenase 5 (ADH5) (99). Most GC-boxes are located near binding sites for other transcription factors, and the Sp/KLF proteins often act in conjunction with other transcription factors to modulate transcription.

Kadonaga *et.al* cloned Sp1 cDNA from HeLa cell RNA and determined the various functional domains using *in vitro* and whole cell assays (100). The structural domains of members of the Sp/KLF family include the N-terminal A domain and the central B domain which contains a glutamine-rich (Q-rich) activation domain flanked by highly conserved serine/threonine-rich regions (Fig. 9). The most highly conserved region is the DNA-binding region which contains three characteristic zinc finger binding motifs encompassed in the C and D domains in the C-terminal region.

Other Sp/KLF members that bind the GC-box include Sp2, Sp3, and Sp4 which are structurally related to each other and Sp1. Sp2 and Sp3 proteins also bind GT-rich elements. Sp1, Sp3, and Sp4 are the Sp proteins with the highest homology containing the characteristic three zinc finger binding motifs, similar activation domains and other structural motifs (101, 102). Other members of the

Sp/KLF family (Sp5, Sp6, Sp7, Sp8, and the Krüppel-like factors) differ from the first four members because they are generally of lower molecular weight and do not contain the Q-rich activation domain in the N-terminal region.

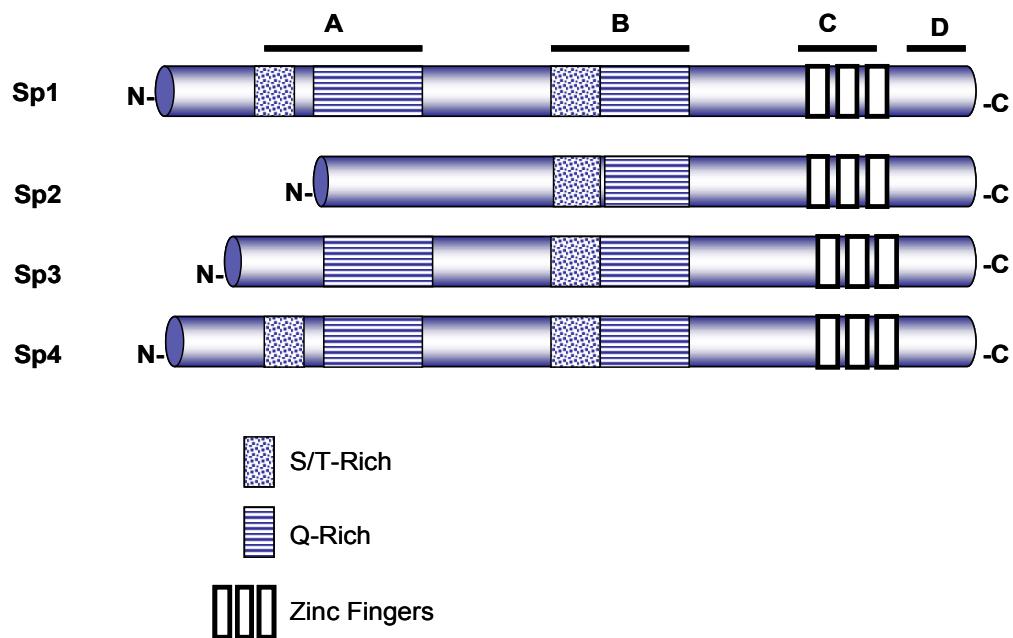


Fig. 9. Members of the Sp family of transcription factors (100).

Sp1 and Sp3 are ubiquitously expressed at high levels in most mammalian cell types and have been implicated in the activation of a variety of genes that are involved in cellular processes such as cell cycle regulation (103, 104), chromatin remodeling (105) and the propagation of methylation-free CpG islands (106, 107), whereas Sp4 expression appears to be restricted to certain cell types of the brain (108, 109). Marin *et al.* reported that knocking out the Sp1 gene in the mouse resulted in embryos that were retarded in development, only

survived until day 9.5 of gestation, and exhibited a broad range of phenotypic abnormalities (110). They also looked at housekeeping, cell cycle-regulated, and tissue restricted genes and found there were no differences in Sp1 expression. Moreover, the CpG islands remained methylated, indicating that Sp1 is essential for preventing methylation of CpG islands (111). This data indicates that Sp1 expression is essential for cell growth and differentiation. In most cases when Sp1 is knocked out, it is assumed that other members of the Sp family such as Sp3 and Sp4 compensate in part for the loss of Sp1 activity in transcriptional regulation of certain genes.

Sp1-dependent activation of gene expression requires interaction with a coactivator complex named CRSP (cofactors required for activation of Sp1). CRSP is a multisubunit complex of six to eight polypeptides that has domains similar to previously characterized polypeptides (112, 113). CRSP is also involved in Sp1-mediated activation of GC-rich genes with TATA boxes or initiator sequences and plays a role in the interactions with proteins of the RNA polymerase preinitiation complex.

Sp1 can directly interact with the TBP (114) and with TATA-binding protein associated factors (TAFs) such as TAF130 (115) and TAF110 (116, 117) and other members of the preinitiation complex through the glutamine (Q)-rich activation domains A and B and with TAF55 through the C-terminal domain (118). In the case of TATA-less promoters, there have to be multiple GC-boxes for Sp1 to activate transcription. Insulin-like growth factor-binding protein-2

(IGFBP-2) gene has a TATA-less promoter and transactivation of the IGFBP-2 promoter can be achieved as long as the three GC-boxes in the minimal responsive promoter region remain intact (119).

Sp1 also interacts with some proteins that are not directly involved in the transcription machinery in order to activate or repress transcription. Most of the proteins that functionally interact with Sp1 are sequence-specific transcription factors. These include ubiquitous factors like Oct-1 (120), NF- κ B (121, 122), and E2F-1 (94, 123). In the murine thymidine kinase promoter, binding sites for Sp1 and E2F are 10 bases pairs apart and interactions between these proteins are cooperative. Sp1 and E2F also interact cooperatively on the dihydrofolate reductase (DHFR) and thymidine kinase promoters to activate expression synergistically. In the DFHR promoter, both proteins are required for synergism. Sp1 also functionally interacts with GATA-1, an erythroid transcription factor (124). Interactions between GATA-1 and Sp1 activate the erythropoietin receptor (EpoR) and chicken α -globin promoters through physical interactions mediated through the zinc finger domains on Sp1.

In some instances, GC-boxes can also function as transcriptional repressor sites as in the case of the human multidrug resistance (MDR1) promoter. Through mutational and deletion analysis, Cornwell and Smith showed that the transcriptionally active region of the MDR1 promoter was a -122 to +1 region relative to the initiation site (125). This promoter sequence contains two GC-boxes that are important for transcriptional regulation. They found that while the

-50 GC-box was involved in activation of transcription, the -110 GC-box was associated with transcriptional repression.

Repression through the GC-boxes can occur through Sp1 DNA binding competition with other Sp proteins. In cases where a promoter contains multiple GC-boxes Sp3 can bind DNA more stably than in promoters with only one GC-box (126). Sp3 binds the GC-boxes as a monomer that slowly forms a complex with other proteins. The resulting Sp3-DNA complex is significantly more stable than monomeric Sp3-DNA complexes as well as multimeric Sp1-DNA complexes. In this case Sp3 becomes a repressor by efficiently displacing Sp1, and preventing Sp1-dependent transactivation.

In other cases, transcriptional repression through Sp1 can occur through protein-protein interactions with other transcription factors (127). For example, FBI-1 is a transcription factor that contains the POZ domain, a protein-protein interaction motif. FBI-1 was shown to physically interact with the zinc finger DNA-binding domain of Sp1 and prevent it from binding the ADH/FDH promoter and subsequently repressing transcription. The interaction of FBI-1 with the zinc finger domain of Sp1 is thought to interfere with the ability for Sp1 to recognize the GC-box.

1.5 Estrogen Receptors

1.5.1 Nuclear Hormone Receptors

The estrogen receptor (ER) is overexpressed in over half of all breast cancer cases, and around 70% of these cases respond to antiestrogen therapy (128). The ER is a member of the nuclear hormone receptor superfamily. Nuclear receptors (NR) are sequence-specific DNA binding transcription factors that comprise a superfamily that includes over 300 known members. Many nuclear receptors bind low molecular weight lipophilic ligands that have the ability to readily cross the plasma membrane and enter the cytoplasm and nucleus (129). A subset of these receptors is called steroid hormone receptors, a nuclear receptor sub-family that binds specific steroids and related compounds and mediates transcription of target genes. Other NRs bind structurally diverse endogenous and/or synthetic ligands and orphan receptors have no known ligands.

The nuclear receptor superfamily can be divided further into subfamilies (Table 1) (130). The first subfamily consists of the thyroid hormone (TR), retinoic acid (RAR), vitamin D (VDR) and peroxisome proliferators-activated receptors (PPAR), as well as orphan receptors. The second subfamily is made up of the retinoic X receptors (RXR) as well as the receptors involved in eye development (TXL and PNR), chicken ovalbumin upstream stimulator (COUP), hepatocyte nuclear factor 4 (HNF4), and testis receptors (TR). The third

superfamily is comprised of steroid receptors such as the estrogen receptor (ER), the progesterone receptor (PR) and related orphan receptors such as the

Table 1. *Mammalian nuclear factor subfamilies (130).*

| | RECEPTOR | SUBTYPE | DENOMINATION | LIGAND | RESPONSE ELEMENT | M, D, H ^a |
|-----------|----------|-------------------------|---|--|---------------------|----------------------|
| CLASS I | TR | α, β | Thyroid Hormone receptor | Thyroid Hormone | Pal, DR-4, IP | H |
| | RAR | α, β, γ | Retinoic acid receptor | Retinoic acid | DR-2, DR-5, Pal, IP | H |
| | VDR | | Vitamin D receptor | Vitamin D | DR-3, IP-9, DR-1 | H |
| | PPAR | α, β, γ | Peroxisome proliferator activated receptor | Wy 14,643, prostaglandin J _s , poly unsaturated fatty acids | DR-1 | H |
| | PXR | | Pregnane X receptor | Pregnanes, C21 steroids | DR-3 | H |
| | CAR | α, β | Constitutive androstane receptor | Androstanes | DR-5 | H |
| | LXR | α, β | Liver X receptor | Oxysterols | DR-4 | H |
| | FXR | | Farnesoid X receptor | Bile acids | DR-4, IR-1 | H |
| | RevErb | α, β | Reverse Erb | Unknown | DR-2, Hemisite | M,D |
| | RZR/ROR | α, β, γ | Retinoid Z receptor/retinoid acid related orphan receptor | Unknown | Hemisite | M |
| | UR | | Ubiquitous receptor | Unknown | DR-4 | H |
| CLASS II | RXR | α, β, γ | Retinoic acid receptor | 9- <i>cis</i> -retinoic acid | Pal, DR-1 | D |
| | COUP-TF | α, β, γ | Chicken ovalbumin upstream promoter transcription factor | Unknown | Pal, DR-5 | D,H |
| | HNF-4 | α, β, γ | Hepatocyte nuclear factor 4 | Fatty acyl-CoA thioesters | DR-1, DR-2 | D |
| | TXL | | Tailles-related receptor | Unknown | DR-1, Hemisite | M,D |
| | PNR | | Photoreceptor-specific nuclear receptor | Unknown | DR-1, Hemisite | M,D |
| | TR2 | α, β | Testis receptor | Unknown | DR-1 to DR-5 | D,H |
| CLASS III | GR | | Glucocorticoid receptor | Glucocorticoids | Pal | D |
| | AR | | Androgen receptor | Androgens | Pal | D |

Table 1. *Continued.*

| | RECEPTOR | SUBTYPE | DENOMINATION | LIGAND | RESPONSE ELEMENT | M, D, H ^a |
|----------|----------------|-------------------------|---|------------|---------------------|----------------------|
| | PR | | Progesterone receptor | Progestins | Pal | D |
| | ER | α, β | Estrogen receptor | Estradiol | Pal | D |
| | ERR | α, β, γ | Estrogen-related receptor | Unknown | Pal, Hemisite | M,D |
| CLASS IV | NGFI-B | α, β, γ | NGF-induced clone B | Unknown | Pal, DR-5 | M,D,H |
| CLASS V | SF-1/FT- F1 | α, β | Steroidogenic factor 1 Fushi Tarazu factor 1 | Oxysterols | Hemisite | M |
| CLASS V1 | GCNF | | Germ cell nuclear factor | Unknown | DR-0d | |

^a Monomer (M), Homodimer (D), Heterodimer (H)

estrogen related receptor (ERR). The fourth, fifth, and sixth subfamilies are made up of orphan receptors such as NGFI-B, SF-1, and GCNF.

There are two estrogen receptor isoforms, classical ER α and the recently discovered ER β (131). ER α and ER β differ in their tissue distribution. ER α is mainly expressed in the epithelial cells of estrogen target tissues such as the uterus, ovaries, and breast tissues (128). ER β is expressed in a wider range of tissue including those of the central nervous system (CNS), cardiovascular, respiratory, gastrointestinal, endocrine, urogenital, musculoskeletal, and reproductive systems (132, 133). ER β expression in these tissues is not limited to the epithelial cells, but can also be detected in nuclei of stromal fibroblasts, endothelial cells, and immune infiltrates.

In the normal mammary gland and during pregnancy ER β is highly expressed in 70% of the epithelial cells and is the dominant ER since there is very little expression of ER α . Low expression of ER α is most likely due to degradation of ER α in response to E2 early during the cell cycle through the ubiquitin-mediated degradation pathway (134, 135). This indicates that proliferation in the normal mammary gland and during pregnancy, one of the most proliferative phases of breast development, is due to an ER α response to E2. However, ER α must be degraded in order for the cell to progress through the cell cycle. ER α is the dominant receptor expressed in 80% of the epithelial cells of malignant mammary tumors (136, 137). Although both ER α and ER β are expressed in the malignant mammary gland, only ER α is responsive to antiestrogen therapy and is therefore the main target for endocrine therapy. In most cases, ER α expression in mammary tumors is an indicator of a positive prognosis (128, 131, 138).

1.5.2 Estrogen Receptor Structure

Members of the nuclear receptor superfamily share a similar structure with different regions corresponding to functional domains that can be interchanged between related receptors. The structure includes a variable A/B region in the NH₂-terminal domain, a C region that contains the DNA-binding domain (DBD), a hinge region D, and a conserved E region that consists of the ligand-binding domain (LBD) in the COOH-terminal domain. Some of the

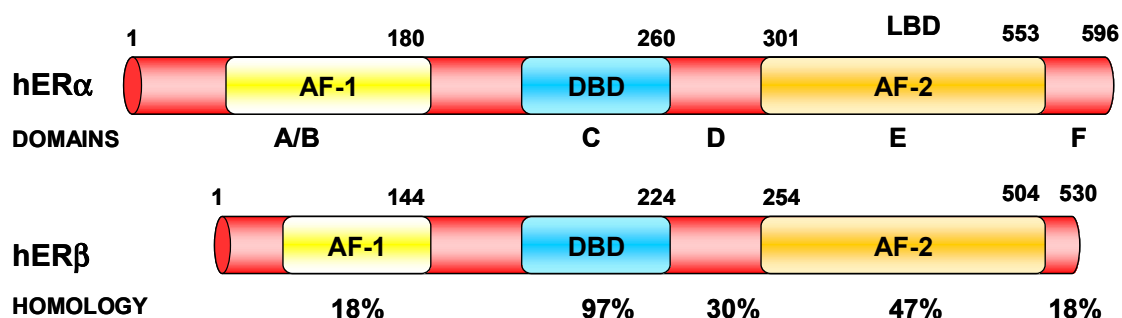


Fig. 10. Domain structure representation of human ER α and ER β isoforms.

receptors also contain an F region whose function is not well characterized (Fig. 10).

The A/B region contains the transcriptional activation function 1 (AF-1) domain and is the most variable region of the receptor. Most receptor isoforms differ mainly in the A/B region. For example, receptor isoforms for the progesterone receptor (A and B), thyroid hormone receptor (TR β 1 and TR β 2) as well as the retinoic acid receptor (RAR α , RAR β , RAR γ) have highly homologous sequences in their DBDs and LBDs, but differ greatly in their A/B regions (139). The AF-1 domain of ER β exhibits 18% homology with the ER α AF-1 domain (Fig. 10) (140). There is also an 80 amino acid difference between the AF-1 domain of the two isoforms and the variability of the AF-1 domain generally results in functional differences between the receptor isoforms. These differences include lower transcriptional activity of ER β compared to ER α in several cell lines, higher binding affinity of ER β for phytoestrogens coumestrol

and genistein, absence of agonistic activity of the antiestrogen 4-OH-tamoxifen on ER β through ERE sites.

Ligand-dependent activation of GC-rich elements through interactions of ER α or ER β with the Sp1 transcription factor was observed only for ER α (139, 141, 142). In domain swapping experiments, it was shown that substitution of the amino-terminal domain of ER β with that of ER α produced a chimeric protein that had the ER α AF-1 domain and the ER β AF-2 domain (ER $_{\alpha/\beta}$). This substitution resulted in improved transactivation of the ER $_{\alpha/\beta}$ chimera in response to E2. Conversely, substitution of the amino terminal domain of ER α with the ER β amino terminal (ER $_{\beta/\alpha}$) resulted in a chimeric protein that had the ER β AF-1 domain and the ER α AF-2 domain (ER $_{\beta/\alpha}$) that produced decreased transactivation. These results indicate that the AF-1 region of ER α is responsible for transactivation through the GC-rich elements. (141, 143).

The AF-1 domain shows promoter and cell type specificity, indicating that it is responsible for modulating the activity of the various receptor isoforms and interaction with cell-specific factors to modulate transcription. It has been shown that the AF-1 domain makes physical and functional contacts with general transcription factors, coactivators and other transcription factors in order to stabilize the preinitiation complex (144-146).

The AF-1 domain is also responsible for ligand-dependent and – independent transcriptional activation or repression. In most cases, activation of

nuclear receptors through the AF1 region is due to phosphorylation mediated by different kinase signaling pathways. For ER α , amino acids 41-150 are required for AF1 activity and amino acids 91-121 are required for interactions of AF-1 with AF2 in LBD. Serines 118, 104, 167, and 122 are targets for phosphorylation through different kinase pathways (MAPK, cyclin A2-CDK2, casein kinase II, and protein kinase C (PKC)- δ respectively) in response to estradiol and growth factors (147-151).

Nuclear receptors regulate gene expression by interacting with specific DNA transcription factors that regulate gene expression and by directly binding to specific DNA promoter sequences of target genes. A two-step mechanism of action was proposed as early as 1968 based on the observation that there were inactive and active states of the receptors (152). The first step involves binding of the ligand to the receptor and the second step involves the binding of the receptor to the DNA and consequently regulating transcription.

The C region contains the DNA binding domain (DBD), and is the most conserved region of all the nuclear receptors. This domain is responsible for recognizing and binding specific response elements on the DNA and activating expression of the gene of interest. The DBD of nuclear receptors usually contains eight cysteine residues as well as two zinc ions that are required for DNA binding. This domain has two zinc finger domains that are each comprised of four cysteines that coordinate tetrahedrally with one zinc ion. These two zinc

finger motifs fold together to form a structure that recognizes and binds DNA (Fig.11).

The zinc fingers of ER α contain a P box at the base of the first zinc finger that is responsible for specific interactions with DNA and contact with the central base pairs of the palindromic response element. The second zinc finger contains the D box that is involved in dimerization (153). The core of the DBD contains two α -helices, the recognition helix that binds the major groove of the DNA and the helix that spans the C-terminus of the second zinc finger forming a right angle with the recognition helix (Fig. 12) (130). ER α and ER β share very similar DBDs (97% similarity) that bind to the same estrogen response element (ERE) (140).

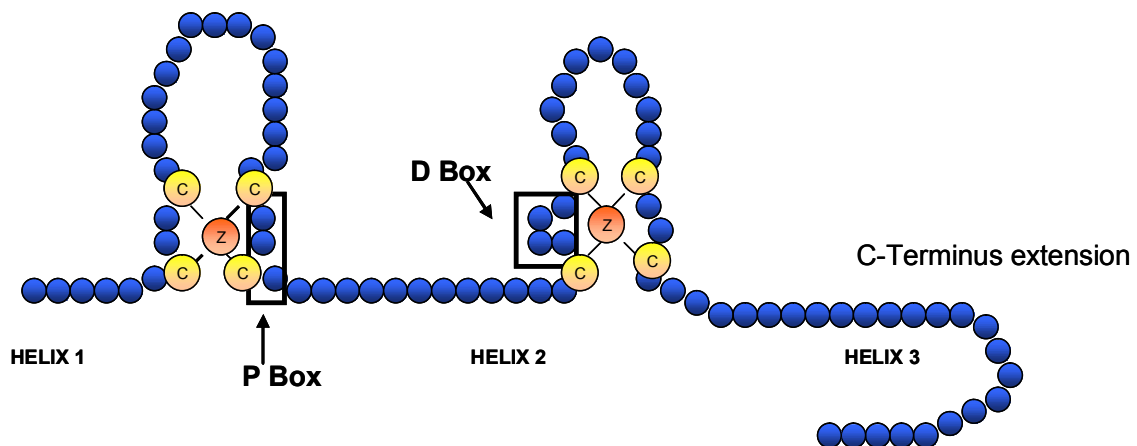


Fig. 11. The zinc finger motif of the DNA binding domain of the nuclear receptors.

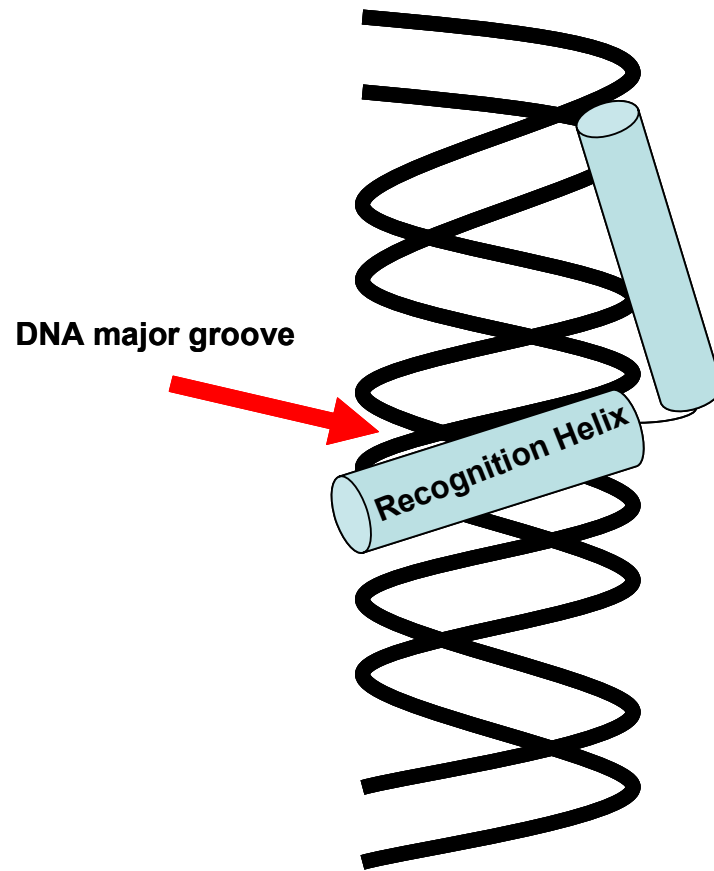


Fig. 12. The DNA binding domain of the estrogen receptor- α .

The D region of ERs is comprised of the hinge domain. The hinge domain is not very well conserved among nuclear receptors, with 30% homology between ER α and ER β (140). This region serves as a linker between the DBD and LBD. In most cases, the hinge domain contains the nuclear localization signals and residues that determine interactions with corepressors. The D region is involved in the conformation changes of nuclear receptors allowing rotation of the DBD upon ligand binding (130).

Most receptors are inactive when not bound to their ligands. In the absence of ligand, the steroid hormone receptors are sequestered by chaperone proteins, such as the heat shock proteins (154). When the ligands bind the ligand binding domain (LBD) within the E region, the nuclear receptor undergoes a conformational change that releases the chaperone proteins and facilitates interaction with specific DNA sequences. The LBD is a multifunctional domain that harbors the hormone binding site, mediates homo- and heterodimerization, and interaction with coactivators and corepressors. Despite low sequence similarity, the three dimensional structures of LBDs are similar for most nuclear receptors. LBDs are formed by 12 conserved α -helical regions numbered H1 to H12 (130). The helices are organized in a three-layered anti-parallel sandwich structure with a center core layer of three helices that create a ligand binding pocket that accommodates the ligand. This cavity is mainly hydrophobic and is buried within the bottom half of the LBD. An anti-parallel β -sheet closes the

ligand pocket on one side and H12 closes another side. The cavity is completely protected from the external environment and buries the ligand in a highly hydrophobic environment.

The ER can form different types of dimers that depend on the bound ligand. The dimerization interface involves helices H8 to H12 interaction with a hydrophobic leucine zipper-like interaction zone and hydrophilic contacts (153). H11 and H12 helices are important for the formation $ER\alpha$ and $ER\beta$ homodimers and for contact with the ligand. This provides a link between ligand binding and dimerization. The binding of an agonist to ER induces a conformational change in the dimer that is different from the antagonistic-bound ER dimer structure (155, 156). Agonist induced rearrangement of ER results in formation of a specific binding site for coactivators at their respective LXXLL motifs that behave as hydrophobic docking motifs that bind the LBD.

$ER\alpha$ and $ER\beta$ can also form heterodimers which bind EREs. The dimeric receptor complex binds DNA and recruits other transcription factors with histone acetyltransferase (HAT) activity that initiate changes in chromatin by altering the nucleosome position and allowing formation of the preinitiation complex (157). Nuclear receptors activate their target genes by stabilizing the preinitiation complex through direct interaction with components of the preinitiation complex including TFIIB, TBP, TFIID, and TFIIF allowing the recruitment of RNAP II to the promoter (158).

1.5.3 Classical Genomic Activation Through the Estrogen Receptor

As indicated above steroid hormone receptor dimers bind specific hexanucleotide palindromic half sites called response elements that are arranged in a particular motif in chromatin and modulate transcription (152, 159). The glucocorticoid response element (GRE) was the first to be discovered in the MMTV promoter (160, 161). The GRE contains two short inverted repeats separated by three nucleotides. The progesterone, mineralocorticoid, androgen and estrogen receptor have similar response elements (Table 2).

Table 2. *Hormone response elements.*

| NUCLEAR RECEPTOR | RESPONSE ELEMENT | DNA SEQUENCE |
|-------------------------|------------------|-----------------|
| Glucocorticoid receptor | GRE | GGTACAnnnTCTTCT |
| Progesterone receptor | PRE | GGTACAnnnTCTTCT |
| Androgen receptor | ARE | GGTACAnnnTCTTCT |
| Estrogen receptor | ERE | GGTCAnnnTGACC |

When bound to their specific DNA sequences, nuclear receptors serve as on-off switches for transcription within the cell nucleus. These switches control various functions including development and differentiation of skin, bone and behavioral centers in the brain, as well as the continual regulation of reproductive tissue, including the mammary gland and breast tumors. There are three features that characterize a response element: the sequence of the base pairs in the half-site, the number of base pairs between the half-sites and the

orientation of the two half-sites. The receptor dimer recognizes the sequence, spacing and orientation of the half-sites within their response element in order to bind to the DNA. Upon binding DNA, the ligand-receptor complex alters the transcriptional level by either activating or repressing of the expression of the associated gene.

There are five classes of nuclear receptors based on their dimerization and DNA binding properties (129). The first class binds DNA as heterodimers with the retinoic acid X receptor (RXR) on directly repeated half sites separated by a spacer of 1-5 base pairs (bp). The second class of receptors binds DNA as heterodimers on inverted responsive sites separated by 1 bp. The third class binds the DNA on direct repeats separated by 1 bp as homodimers. The fourth class, to which ER belongs, also binds DNA as homodimers on inverted repeats separated by 3 bp (consensus ERE: 5'-GGTCAnnnTGACC-3'). The fifth class binds DNA as monomers on a single half site often containing a 3bp 5' extension. Although a few estrogen target gene promoters contain perfect palindromic ERE sites that match the consensus sequence, most of the identified elements are imperfect or nonconsensus. While the ER-binding affinity for imperfect EREs is reduced, these sequences are functional in mediating transcriptional activation of reporter genes (162, 163).

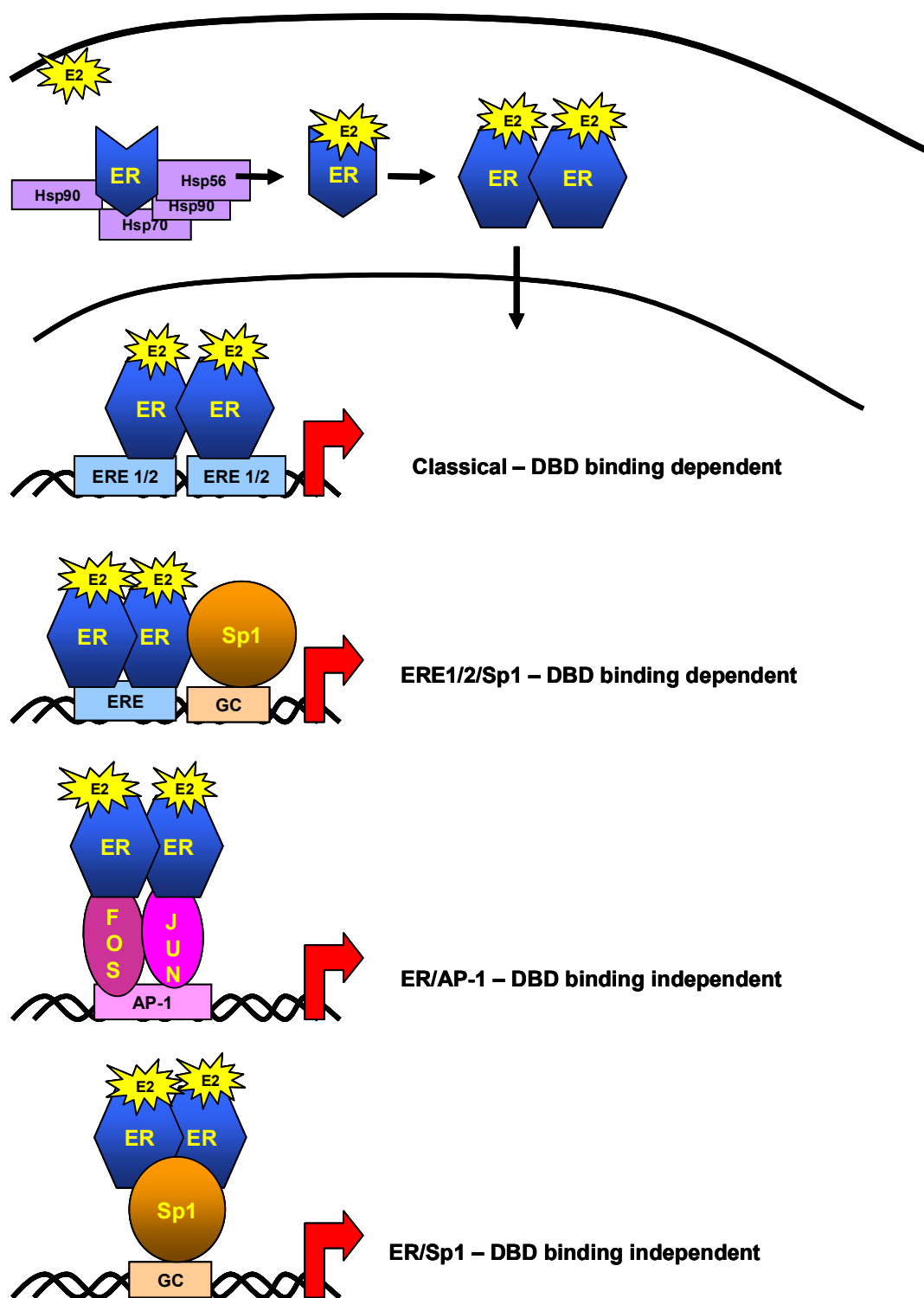


Fig. 13. Molecular mechanisms of ER genomic action.

1.5.4 Non-Classical Genomic Activation of the Estrogen Receptor

1.5.4.1 ERE_{1/2}-Sp1 Interactions

In addition to the classical ER-ERE interactions, there are other DNA-binding dependent pathways that are important for regulation of estrogen-responsive genes. One of these mechanisms involves the transactivation of genes containing an ERE half site associated with GC-rich elements that bind the transcription factor Sp1 (Fig. 13). The distance and the orientation of the half ERE and Sp1 motifs can be variable, and the sequence of the ERE can be imperfect. For example, an ERE half site and GC-rich element (Sp1(N)₂₃-ERE) were shown to be important for the transactivation of the cathepsin D gene promoter. Mutations of the ERE half site or the Sp1 sites rendered the promoter no longer inducible by estrogen (164, 165).

Subsequent studies have revealed that Sp1(N)_xERE motifs are important in the transactivation of other gene promoters including the retinoic acid receptor (RAR) α -1 and the heat shock protein 27 promoters (166, 167). Cooperativity with Sp1 has also been reported for promoters containing consensus EREs such as the vitellogenin A1 and rabbit uteroglobin promoters (168, 169). Other studies have also shown that Sp1(N)_xERE motifs may function as response elements for other estrogen regulated nuclear receptors including the progesterone receptor (PR). Promoter A of the PR gene contains an ERE half site upstream of two adjacent Sp1 sites. In this case, mutation of the ERE half

site increases transcription substantially suggesting that this ERE plays a limiting role in transcription (170). The testes receptor (TR)-4, an orphan receptor, activates the luteinizing hormone receptor gene through an ERE half site located 50 base pairs (bp) upstream of an Sp1 site, whereas other orphan receptors EAR2 and EAR2/COUP-TFI repressed expression of the same gene (171). Sp1 has also been shown to mediate DNA-binding dependent transactivation with other transcription factors (94, 123)

1.5.4.2 ER/Sp1 Interactions

There is also increasing evidence that ER can function cooperatively with Sp1 even in the absence of an ERE (Fig. 13). Porter *et.al* identified an Sp1(N)₁₀ERE_{1/2} motif that forms a complex with Sp1 and ER proteins in the 5' promoter region of the heat shock protein 27 (Hsp 27) gene (167). Further analysis of this region revealed that upon mutation of the ERE_{1/2} site, estrogen responsiveness of the Hsp 27 gene was not lost in transient transfection studies. Gel shift mobility studies showed that Sp1 protein formed a protein-DNA complex with wild type and mutant Sp1(N)₁₀ERE_{1/2}, and consensus Sp1 oligonucleotides. It was also shown that incubation of these complexes with wild type ER or an ER mutant with a DNA-binding domain deletion enhanced the intensity of the protein-DNA retarded band complex in gel shift mobility assays.

Immunoprecipitation studies revealed that there were protein-protein interactions between ER and Sp1 proteins, and these interactions were observed in the presence or absence of E2. These data indicate that there is

functional cooperativity between Sp1 and ER that facilitates transactivation on the Hsp 27 promoter. This interaction occurs through Sp1 binding to the GC-box, but not through the ERE_{1/2} site. These studies identified a new estrogen-dependent transactivation pathway that did not require ER binding to the DNA, but rather Sp1 protein which bound to the GC-box while interacting with ER (172). There are other genes with GC-boxes that can be activated through an ERE-binding independent manner. Some of these include *c-fos* (173), adenosine deaminase (174), *bcl-2* (175), thymidylate synthase (176), cyclin D1 (177), and *cad* (178).

Saville *et.al* have shown that both ER α and ER β interact with Sp1 through the C-terminal domain of Sp1 (141). E2 enhanced ER α /Sp1- but not ER β /Sp1-dependent transactivation in breast and prostate cancer cell lines. Exchange of the AF domains of ER α and ER β proteins produced chimeric proteins ER α / β and ER β / α that were able to associate with the Sp1 protein. Only the ER α / β (not ER β / α) chimera was able to activate transcription of a GC-rich promoter. This indicates that the AF-1 domain of ER α is important for transcriptional activation at GC-boxes suggesting that coactivators recruited by the AF-1 region play an important role in ER α /Sp1- dependent transactivation.

Regulation of ER in the absence of DNA-binding is not limited to ER/Sp1 interactions. ER/AP-1 interactions are another extensively investigated ERE-independent pathway (Fig. 13). ER-dependent activation of genes with AP-1

elements can be observed for the chicken ovalbumin, human IGF-1, or human collagenase genes (179). In most cases, ER activation mediated by AP-1 can be observed with ER mutants lacking the DNA-binding domain (180). Fos and jun are components of the AP-1 complex that bind to the AP-1 DNA element. Interactions between ER and AP-1 have been shown to be ER activation function-dependent and independent depending on the receptor, ligand, and cell type (181). ER α can also repress transcription in the absence of DNA-binding and E2-induced transactivation of erythropoiesis is inhibited by ER interactions with GATA-1 (182).

1.5.5 Membrane ER

1.5.5.1 Identification

It has been observed that for some responses the effects elicited by steroid hormones are too rapid to be mediated by nuclear ER α -dependent transactivation. These observations have prompted the search for alternate pathways to the classical and non-classical genomic ER signaling pathways. In 1977, Pietras and Szego described the presence of cytoplasmic membrane binding sites for estradiol in endometrial cells (183, 184). They described an E2 binding protein, presumably ER, in the cell membrane that triggered rapid generation of cAMP. Subsequent work in the 1980s and 1990s demonstrated that estrogen could rapidly activate signaling such as calcium flux (185), cAMP generation (186), phospholipase C activation (187), and inositol triphosphate

generation (188), leading to the activation of protein kinase C (PKC) and protein kinase A (PKA) activation (189, 190). Research on mERs has provided evidence that they are involved in alternative ER signaling pathways (190). These pathways have been called non-genomic or extranuclear pathways. It has also been suggested that the term non-transcriptional may be used indicating that DNA binding of the receptors is not required.

To date, the endogenous membrane ER has not been isolated and therefore the structure of mER has not been determined. However, Pappas *et al.* used a variety of antibodies directed against multiple epitopes of ER α and identified an endogenous membrane protein in several cell lines (191). In 1999, Razandi *et al.* addressed this issue by transfecting Chinese hamster ovary cells, which do not normally express ER, with the cDNA for ER α and ER β (192). Results of this study led to the conclusion that membrane ERs (mER) originate from the same transcript as nuclear ER (nER) and have near-identical affinities for 17- β -estradiol. This was also the first study to identify a membrane ER β .

1.5.5.2 Membrane Localization

Growth factors receptors, G-protein coupled receptors (GPCR) and non growth factor tyrosine kinases are examples of membrane receptor signaling molecules. Signaling through these molecules occurs in part after localization to plasma membrane microstructures known as caveolae (193, 194). Caveolae facilitate signal transduction through the localization of signaling molecules (195). Within the caveolae, signaling molecules interact with caveolins, members of a structural coat protein family. Caveolin-1 serves a scaffold protein that associates with and activates a variety of signaling proteins in caveolae (195). Recent studies have shown that ER localizes mainly to caveolae and non-caveolar fractions of endothelial cells (196, 197) indicating that membrane ERs behave in a similar manner to other membrane receptor signaling molecules.

1.5.5.3 Mechanisms of mER Action

Generally, it is thought that in order for the effects of a hormone to be considered to be non-genomic, the actions of the hormone must be too rapid to be compatible with the time it would take to induce RNA and protein synthesis. The action of the hormone should also be reproducible in the presence of inhibitors of RNA and protein synthesis, further indicating that these processes are not required for the hormonal effects (198). This response usually occurs within seconds to minutes after exposure to the hormone as opposed to the genomic response that occurs within hours after hormone addition. However,

there are intermediate responses that occur several hours after treatment with E2, and these responses are not persistent. In these cases, the recruitment of non-genomic pathways regulates longer-term processes including gene expression, cell proliferation, protein and DNA synthesis. This response involves crosstalk between genomic and non-genomic responses and is called a membrane-initiated response due to the fact that the hormonal response involves membrane protein activity associated with surface receptors such G-protein coupled receptors (GPCR), growth factor receptors, ion channels, or membrane associated signaling complexes (199, 200).

Other hormones besides E2 are also important for breast cancer growth. Growth factors bind to their plasma membrane bound growth factor receptors and initiate kinase-signaling cascades that are responsible for the sequential phosphorylation and activation of a series of intracellular signaling molecules (Fig. 14). There are several important pathways that are activated by growth factors including the phosphatidylinositol-3-kinase (PI3-K) and AKT/PKB pathway (201, 202) and the mitogen activated protein kinases (MAPK) ERK1 and ERK2 (203, 204). These complex pathways have been shown to independently modulate ER activity through phosphorylation (205-207).

The PI3K/AKT pathway is involved in the mediation of cell survival and proliferation signals from a variety of growth factors including insulin, the insulin-like growth factors (IGF), and members of the epidermal growth factor (EGF) family (202). PI3K/AKT modulates ER by phosphorylation of the serine 167 and

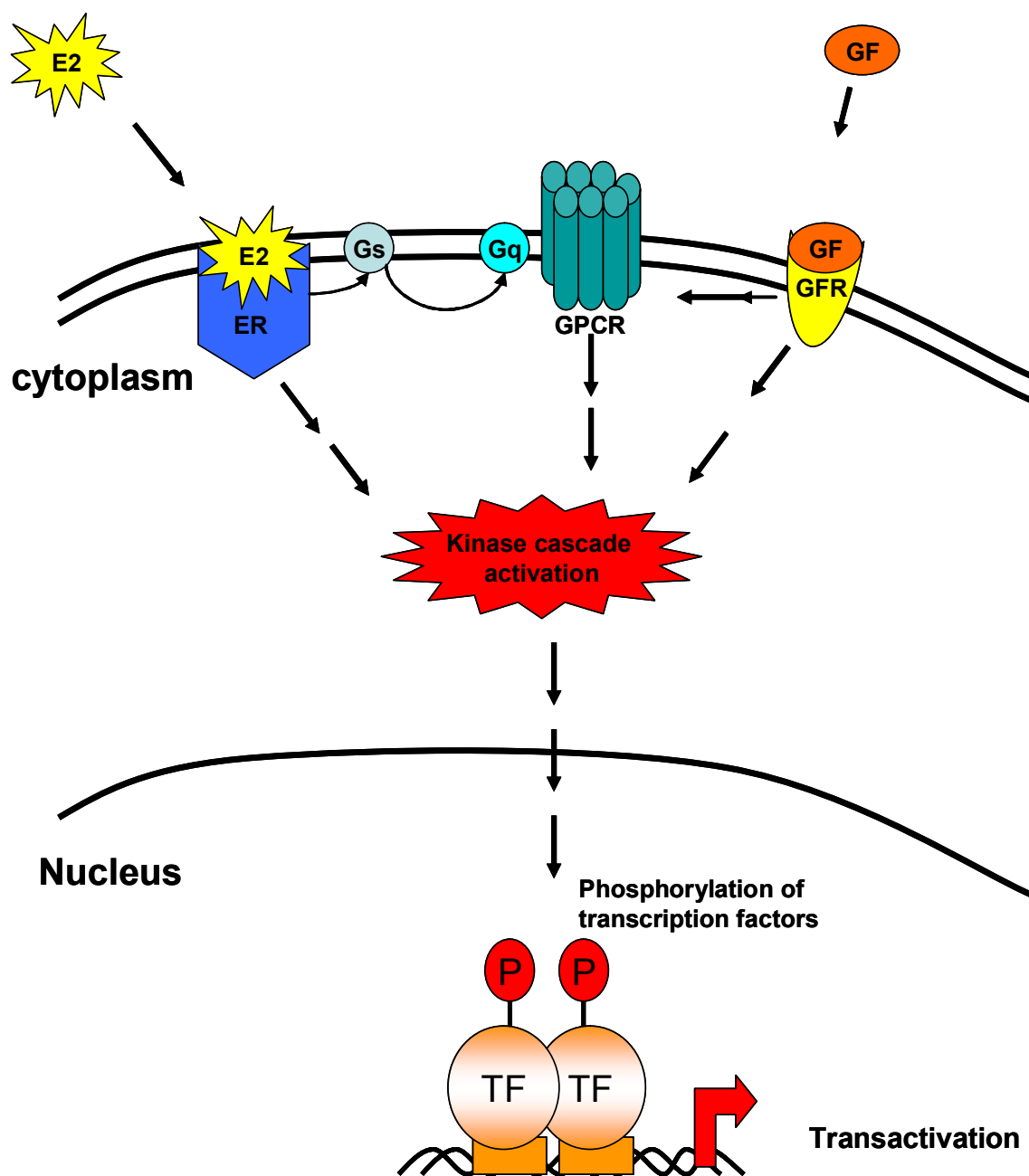


Fig. 14. Non-genomic ER α -mediated transactivation. GPCR (G protein coupled receptors), GF (growth factor), GFR (growth factor receptor), TF (transcription factor), P (phosphorylation).

118 residues of ER (151, 208, 209). Phosphorylation of the serine residues activates ER and allows it to be transcriptionally active.

IGF-1 induces cell proliferation and/or DNA synthesis of ER-positive breast cancer cells and these responses have been shown to be inhibited by PI3K inhibitors such as wortmanin and LY294002, as well as antiestrogens, demonstrating that the mitogenic activity of IGF-1 is linked to regulation of estrogen-responsive genes. Xie *et al.* showed that IGF-1 induced expression of the adenosine deaminase (ADA) gene in MCF-7 cells was mediated through activation of ER α /Sp1 (210). IGF-1 induced both MAPK and PI3K phosphorylation cascades. However, only the MAPK inhibitors inhibited transactivation of ADA gene promoter constructs by IGF-1, indicating that activation of the ADA gene in MCF-7 cells is dependent on the MAPK signaling pathway (210).

There is also evidence of ER-growth factor receptor crosstalk. E2 has been shown to induce uterine cell proliferation through the actions of EGF (211). This might occur through mER utilization of the EGF receptor as a signal transduction scaffold molecule to effect signaling. E2 also increases expression of several growth factors and their receptors to amplify signals generated through these pathways (212, 213). Therefore growth factor activation of ER and E2 activation of growth factor receptors act together to allow crosstalk between ER and

growth factor receptors to synergistically activate genes in breast cancer cell lines.

G-protein coupled receptors (GPCR) are a large group of molecules located in the plasma membrane. GPCRs have been recognized as mediators of multiple extracellular stimuli including those induced by E2. For example, it has been reported that E2 induces ERK phosphorylation through GPR30, a GPCR homologue (214). Studies in this lab have reported that the transcription of *c-fos* is controlled through the serum-response element that binds the serum-response factor which recruits Elk-1 and the serum response factor accessory protein 1 and 2 to mediate induction by growth factors and other extracellular stimuli leading to activation of MAPK pathways (215). Duan *et al.* reported that activation of *c-fos* by E2 involved non-genomic signaling of ER α through the MAPK pathway and phosphorylation and binding of Elk-1 to the serum-response element in ER α -positive MCF-7 breast cancer cells (215). A study by Maggiolini *et al.* reported that E2 activation of *c-fos* through the MAPK pathway in MCF-7 cells as well as ER α -negative SKBR3 breast cancer cells involved stimulation through GPR30 in an ER α -dependent and –independent manner (216).

ER binding is required in most cases where E2 induces a non-genomic response. Studies in CHO cells showed that E2 bound to ER α or ER β and expression of these mER proteins activated G α q and G α s proteins (Fig. 14) (192). This study provided that first direct evidence that mERs are linked to G-

proteins. Since these non-genomic signaling responses occur through activation of G proteins by E2, this qualifies mER as a member of the GPCR family (192).

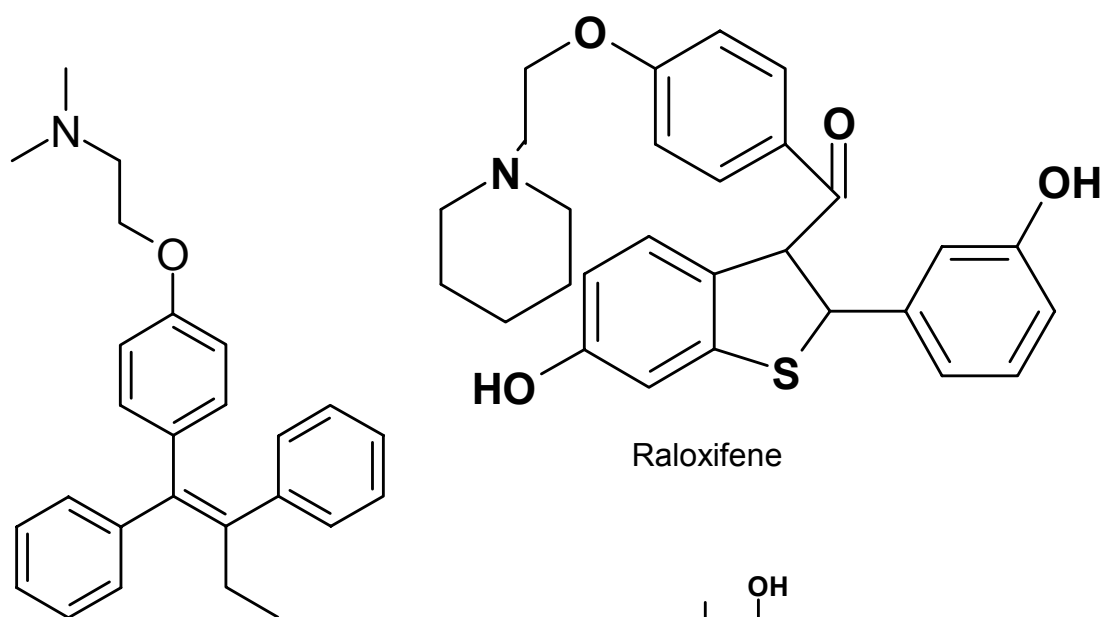
Membrane ER activation of kinase cascades integrates the membrane and nuclear actions of estrogen. E2 treatment has been shown to stimulate a variety of kinase cascades in mammary and prostate cancer cells that are responsible for phosphorylation and activation of other kinases and nuclear transcription factors including the ELK-1 of *c-fos* gene in breast cancer cells (215). Qin *et al.* reported that E2 induced p53 gene expression through E2-dependent phosphorylation of calmodulin-dependent protein kinase IV (CaMKIV) (217). Other studies in this lab have shown that E2 induces the activation of the protein kinase A pathway that in turn phosphorylates transcription factors at the gene promoter to promote transactivation. The cyclin D1 and ornithine decarboxylase gene promoters are both activated through multiple enhancer elements. The cyclin D1 gene expression is induced by E2 through ER α /Sp1 genomic activity as well as non-genomic activation involving phosphorylation through the cAMP-response element (CRE) in ZR-75 breast cancer cells (177). The ornithine decarboxylase (ODC) gene is also regulated through PKA activation by E2 in MCF-7 cells. PKA activation of the ODC gene occurs primarily through the CCAAT sites that bind NFYA and the LSF sites that bind LSF (218). Recently Li *et al.* reported that the lactate dehydrogenase A gene was regulated by E2 through activation of CRE through protein kinase C (PKC)

(219). These studies have shown that E2 can induce non-genomic responses that activate genomic transcription factors in order to mediate transactivation of gene promoters in MCF-7 and ZR-75 breast cancer cells.

1.5.6 Selective Estrogen Receptor Modulators

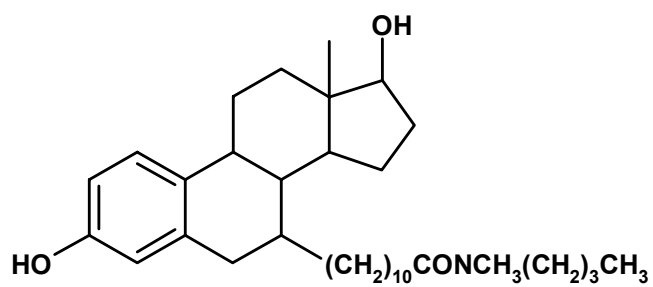
Increasing evidence has shown that although estrogens are associated with an increased incidence of breast cancer, steroid hormones are essential for many tissue-specific homeostatic responses and hormone replacement therapy is routinely used for treating postmenopausal problems such as osteoporosis. This has led to development of tissue-specific ER agonists/antagonists that have been designated as selective estrogen receptor modulators (SERM). These compounds were first generated in the 1950s as fertility agents. The beneficial effects of antiestrogens in breast cancer patients became apparent shortly after they were generated. However many of the compounds which included stilbene analogs also induced significant toxicity (220). The first studies of the effects of antiestrogens on breast cancer patients were published in the early 1970s (221, 222). This study introduced a new SERM called tamoxifen (ICI 46,474) which 30 years later has become the primary treatment for early stage breast cancer in women and a chemopreventive agent for women at high risk for this disease (223-229).

Antiestrogens are classified into two groups according to their mechanism of action. Type I antiestrogens include compounds that are agonistic as well as antagonistic, while the type II group includes “pure” antiestrogens that are

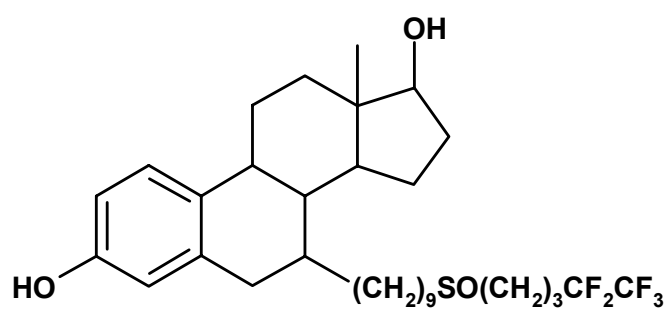


Tamoxifen

Raloxifene



ICI 164,384



ICI 182,780

Fig. 15. Selective estrogen receptor modulators (SERMs).

primarily ER antagonists. Type I antiestrogens affect ER function in part by affecting AF-1 and DBD through antiestrogen-induced conformational changes that are different from those induced by E2 (141, 230-233). These different ligand-induced conformations allow the ER to interact with different sets of cofactors that determine whether the effects will be agonistic or antagonistic (234). Type II antiestrogens may affect ER function by blocking dimerization (235, 236), disrupting nuclear localization of ER (237), and inducing rapid degradation of ER in cultured cells or *in vivo* (238, 239).

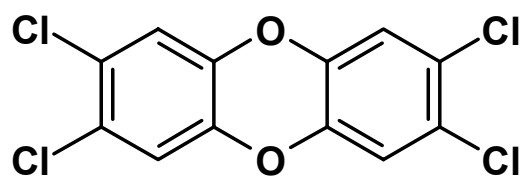
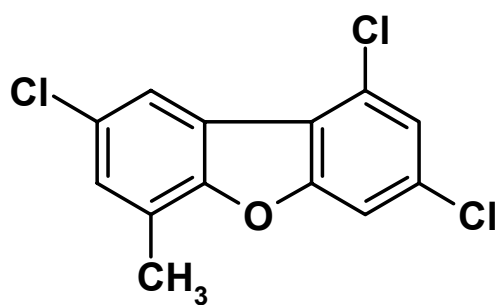
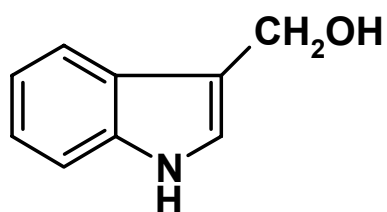
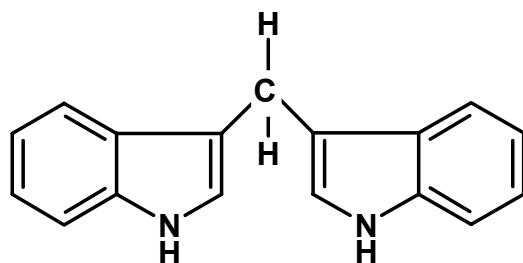
Tamoxifen (Fig. 15) is an example of a type I antiestrogen. Tamoxifen has been proven to be effective for prevention and treatment for breast cancer resulting in a 50 % decrease in the incidence of new breast cancer for up to five years (240-242). Unfortunately, prolonged treatment with tamoxifen results in tamoxifen resistant tumors (243-245). It has been shown that although tamoxifen acts as an antagonist in the breast, it exhibits partial estrogen agonist activity in the rodent uterus (246). Several studies have shown that tamoxifen causes a low but significant increase in the incidence of endometrial cancer in women undergoing tamoxifen treatment for over five years and similar results have been reproduced in *in vitro* and *in vivo* (247-249). In contrast tamoxifen exhibits estrogenic activity in the bone and decreases osteoporotic fractures by maintaining bone density in postmenopausal patients (250).

Ongoing studies have been focused on developing SERMs that will maintain bone density while preventing and inhibiting breast cancer

development without causing endometrial cancer. Results of these studies have identified raloxifene (Fig. 15), a SERM that was originally discarded as a breast cancer drug. Raloxifene was shown to maintain bone density in ovariectomized rodents as well as decrease the incidence of mammary tumors (251, 252).

These results have also been observed in clinical studies where osteoporotic fractures and mammary tumors were decreased by raloxifene in women at risk (253). Currently, raloxifene is available as a preventive treatment for osteoporosis and applications of this drug for treatment of breast cancer will be forthcoming.

Type II antiestrogens have been termed “pure” antiestrogens because they do not possess partial agonist activity for ER. ICI 164,384 (Fig. 15) was the first antiestrogen to be characterized as an ER antagonist with no discernible agonistic effects (254). ICI 182,780 (Fig.15) was found to be the most potent and among the more promising new antiestrogens for the treatment of breast cancer (232). Unlike tamoxifen, these pure antiestrogens exhibit antagonistic effects on mammary tumors and are devoid of uterotrophic effects; however, they lack the beneficial agonistic effects in the bone. Treatment with either ICI 164,384 or ICI 182,780 resulted in the inhibition of estrogen responsive genes in the breast and uterine tissue (164, 255-261). However, in some cases the pure antiestrogens behaved as ER agonists rather than that ER antagonists and there are some genes that are inducible by ICI 182,780 and estrogens (262).

**2,3,7,8-TCDD****6-MCDF****Indole-3-carbinol
(I3C)****Diindolymethane
(DIM)****Fig.16. Selective aryl hydrocarbon receptor modulators (SAHRMs).**

1.6 Aryl Hydrocarbon Receptor

1.6.1 AhR-Mediated Carcinogenesis or Anti-carcinogenesis

The aryl hydrocarbon receptor (AhR) is a ligand-inducible transcription factor that was first identified by Poland *et al.* in 1976 in the hepatic cytosol of mice using [³H]-2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Fig. 16) as a radioligand (263). The AhR plays a role in the response to certain foreign chemicals including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). TCDD is the most toxic member of the polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofuran (PCDF) family of chemicals that are formed as by-products of industrial processes and during combustion (264). TCDD has been used as a prototype to study the mechanism of action of this group of compounds. PCDDs and PCDFs are also byproducts in some herbicides and pesticides, and due to their widespread use and environmental persistence, these compounds are routinely detected as contaminants in fish, wildlife, human adipose tissue, serum and milk (265).

Individuals most highly exposed to PCDDs/PCDFs include industrial workers involved in the manufacture of products that contain these compounds as contaminants, herbicide sprayers, and several groups that were accidentally exposed to these compounds. The most famous exposure to PCDDs/PCDFs was associated with their presence in Agent Orange, a herbicide used in Vietnam to kill the thick vegetation so that the U.S armed forces could better

detect their enemies. This massive exposure resulted in unwanted side effects in members of the U.S armed forces as well as the Vietnamese population that included immunosuppressive effects, hepatotoxicity and porphyria, tumor promotion, hyperplastic effects, reproductive and developmental effects, neurotoxicity, chloracne and other dermal effects (266).

Although in recent years there has been much debate about the toxicity of TCDD, in 1997 the International Agency for Research on Cancer (IARC) concluded that TCDD was a human carcinogen (264). This was determined from animal studies and the increased cancer mortality rates in industrial workers who were exposed to dioxins. Several studies showed that TCDD is not genotoxic and is more likely a tumor promoter. Kociba *et al.* reported that TCDD induced liver tumors in female Sprague-Dawley rats but not in male rats (267). In contrast, dietary exposure to TCDD decreased the rates of spontaneous mammary and uterine tumors in female Spague-Dawley rats suggesting that TCDD may be antiestrogenic in these two E2-responsive tissues (268-271).

TCDD and other AhR ligands including 6-methyl-1,3,8-trichlorodibenzofuran (MCDF, Fig. 14) and diindolymethane (DIM, Fig. 14) inhibit 7,12-dimethylbenzanthracene (DMBA)-induced growth. DMBA is a chemical used to induce mammary tumors in female Sprague-Dawley rats (269, 271, 272). A study conducted by Holcomb *et al.* indicated that in rats treated with a carcinogenic dose of DMBA in order to stimulate mammary tumors, TCDD decreased tumor volumes and in some cases small tumors disappeared after 3

weeks of TCDD treatment compared to the control group treated with the corn oil vehicle (271). 6-MCDF is a non toxic analog of TCDD that has been developed for potential use as treatment for breast cancer and has been found to significantly inhibit tumor growth in the DMBA-mammary tumor model (272, 273). Other TCDD analogs are derivatives of indole-3-carbinol (I3C) (Fig. 16), a component of brassica vegetables. I3C itself weakly binds AhR. However in the acidic environment of the intestinal gut, I3C metabolites, including DIMs, exhibit higher binding affinity for AhR (264). Several studies have indicated that I3C and its metabolites can inhibit mammary tumor formation and growth by inhibiting ER activity (269, 274-277).

AhR agonists also inhibit E2-induced responses in the rat uterus. TCDD decreased E2-induced nuclear and cytosolic ER levels, peroxidase activity, EGF receptor, and c-fos mRNA levels in the rat uterus (278-281). Treatment with TCDD also decreased E2-induced uterine weight changes and uterine ER levels in mice (282, 283).

1.6.2 Inhibitory AhR/ER Crosstalk

TCDD and related compounds have been used to study AhR-mediated responses in ER-positive and ER-negative human breast cancer cell lines. Gene expression studies indicate that treatment with AhR agonists increased the expression of CYP1A1 mRNA and protein in ER-positive and some ER-negative breast cancer cell lines (284). The ER-positive MCF-7 and T47D breast cancer cell lines express functional AhR and treatment of these cells with

TCDD induced CYP1A1 gene expression (285-287). Moreover, in cells cotreated with E2 plus TCDD there was significant inhibition of E2-induced cell proliferation (287-290), secretion of tissue plasminogen activator (288), cathepsin D secretion and mRNA (291), progesterone receptor (PR) mRNA and protein levels (292) and downregulation of ER protein (290, 293-295). TCDD was also found to inhibit E2-induced cell cycle responses, enzymes and also blocked hormone-induced G₁ to S progression in breast cancer cells (296).

1.6.3 AhR and Arnt Structure

The AhR and AhR nuclear translocator (Arnt) proteins are members of the basic helix-loop-helix (bHLH) family of transcription factors that contain several domains which are responsible for DNA binding, ligand binding, and interaction with other proteins that are required for ligand-induced transactivation (Fig. 17). The bHLH region is found in the N-terminal region of the AhR protein and is responsible for dimerization with the Arnt and DNA binding. The HLH region is mainly responsible for interactions with Arnt, while the basic region is required for interaction with DNA. Deletion analysis revealed that both the α -helices in the bHLH region are required for dimerization with Arnt and DNA binding (297).

Arnt was first identified as the non-ligand binding component of the transformed AhR DNA binding complex (298-300). In 1991, Arnt was cloned and sequenced and was found to be structurally similar to AhR (Fig. 17). Arnt also possesses a bHLH domain in the N-terminal region, a PAS domain, and a

transactivational domain in the C-terminal region of the protein (301-303) . Like the AhR protein, both α -helices in the bHLH region of Arnt are required for dimerization with AhR and the basic region is required for DNA binding (304). Subsequent studies have shown that Arnt (also known as hypoxia inducible factor-1B (HIF-1 β)) forms a heterodimer with HIF-1 α to mediate hypoxia-inducible gene expression. Arnt is also a heterodimeric partner for many other bHLH genes (305, 306).

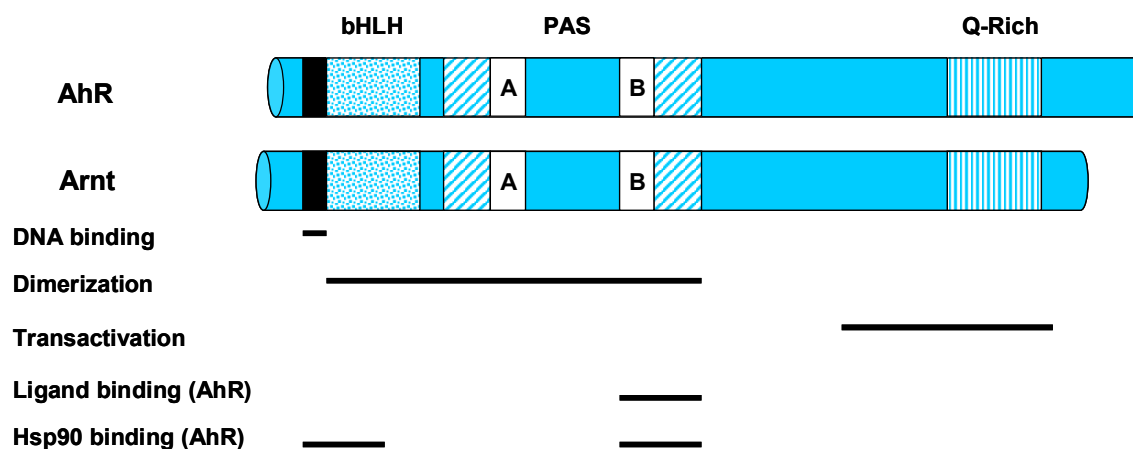


Fig. 17. Structural and functional domains of AhR and Arnt (237).

AhR and Arnt both contain a PAS (Per-Arnt-Sim) domain downstream of the bHLH region that is necessary for interactions with each other (307). PAS domains are also found in the *Drosophila* protein period (Per) mammalian Arnt and single-minded (Sim) and this domain mediates protein dimerization (308,

309). The PAS domain contains two imperfect 51 amino acid repeats that are referred to as PAS A and PAS B. While the rest of the protein structures between AhR and Arnt are similar, their PAS domains are functionally different. In the inactive form, the AhR interacts with two heat shock proteins 90 (Hsp90) in the cytosol. One of the Hsp90 proteins interacts with the PAS B domain while the other Hsp90 protein interacts with the bHLH region (297, 301, 310, 311). The PAS B domain is also required for ligand binding and DNA binding activities of the AhR. In contrast, the PAS B domain of Arnt does not bind ligand or Hsp90 (297, 312-317).

In most species, the N-terminal region of the AhR is highly homologous. Most of the differences occur in the C-terminal region that contains multiple transcriptional activation domains, including the glutamine (Q) rich domain and a reversible domain that specifically represses transactivation by AhR and Arnt in the absence of ligand (301, 303, 318, 319). The transactivation domains of AhR and Arnt are also functionally different. Transactivation by Arnt requires the intact protein while the C-terminal region of AhR is sufficient for transactivation in the presence ligand and Arnt.

1.6.4 The Role of AhR and Arnt in Mammalian Development

AhR null mice have been generated independently by several groups in order to provide information on the role of the AhR in mammalian development. Fernandez-Salguero *et al.* observed 40-50% mortality of AhR knockout pups in the first two weeks (320). These studies indicated that in animals that do not

express the AhR there is reduced liver size, decreased growth rate over the first four weeks, and resistance to induction of CYP1A1 and CYP1A2 gene expression by TCDD and TCDD-induced toxicities (320-322). Overall, the AhR knockout mice were viable suggesting that the AhR either serves a redundant function or is not essential to life (320, 323). In contrast, the generation of Arnt deficient mice resulted in the death of mice *in utero* prior to day 10.5 of gestation and this may be related to the critical role of Arnt in regulating expression of genes during hypoxia (324, 325).

1.6.5 Mechanism of AhR Mediated Transactivation

The unliganded Ah receptor is generally found in the cytosol in a 290- to 300-kDa complex that has low DNA binding affinity and contains the heat shock 90 protein and a novel protein known as AIP (AhR-interacting protein) at low temperature. The Hsp90 proteins serve as chaperones that keep the AhR in the cytosol in its inactive form. Upon increase of temperature or treatment with TCDD the AhR is dissociated from the heat shock proteins and is associated with the Arnt protein to form a 180- to 200-kDa complex that is then translocated into the nucleus. This AhR/Arnt complex exhibits a higher affinity for DNA binding than the AhR/Hsp90 complex.

Most initial studies on the molecular biology of AhR action were determined by analysis of the 5'-flanking region of the CYP1A1 gene. The result of these studies identified *cis*-acting genomic dioxin or xenobiotic responsive elements (DRE/XRE) that interacted with the AhR/Arnt complex resulting in transcriptional

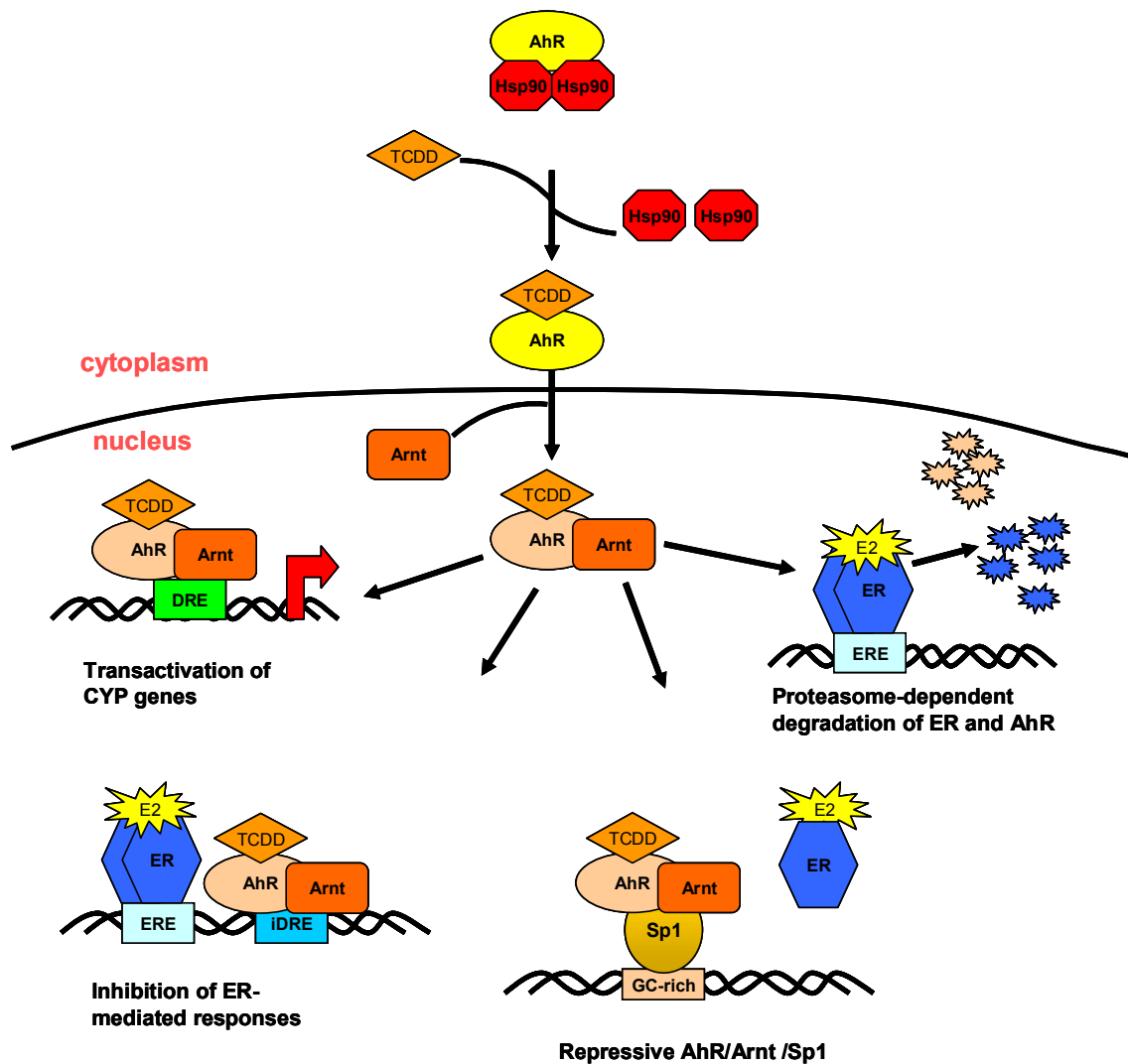


Fig. 18. Mechanisms of AhR transactivation and inhibition of ER α -mediated responses.

activation of the CYP1A1 gene. Although the AhR is not a member of the steroid hormone nuclear receptor family, its transactivation is similar to that of steroid receptors in that a heterodimer must be formed in order to bind to the responsive element in the nucleus (Fig. 18). Promoter regions of many genes contain functional DRE/XREs (consensus sequence TNGCGTG) that bind the ligand-activated AhR complex and some of these Ah-responsive genes include CYP1A2, CYP1B1, glutathione S-transferase, and aldehyde-3-dehydrogenase (270, 326, 327).

1.6.6 Mechanisms of Inhibitory AhR-ER α Crosstalk

In most cases where genes such as CYP1A1 are activated by the Ah receptor, the AhR/Arnt complex interacts with a dioxin response element located in the gene promoter. Binding of the AhR/Arnt heterodimer to the DRE/XRE remodels the chromatin structure and facilitates the association of other transcription factors in the promoter region in order to activate transcription (328). However there are instances where AhR/Arnt agonists act as inhibitors rather than activators of ER α -mediated gene expression (262, 268, 329-334). Studies in this laboratory have identified inhibitory dioxin responsive elements (iDRE) that would account for the inhibitory mechanism of AhR-ER α crosstalk (Fig. 18). TCDD inhibited E2-induced secretion and transcription of the cathepsin D gene in MCF-7 cells (165, 289, 335). The cathepsin D promoter contains an estrogen-responsive ERE_{1/2}(N₂₃)Sp1 motif that is inhibited by

cotreatment with TCDD. Examination of the sequence between the ERE half-site and the GC-rich Sp1 binding sites revealed the presence of an overlapping core inhibitory DRE motif (iDRE). Mutation of the DRE motif had no effect on reporter gene activity induced by E2 or DNA binding in gel shift mobility assays. However, TCDD was no longer able to disrupt E2-induced responses (165). The AhR/Arnt complex interacts with the iDRE on the 5'-flanking region of many other genes including, *c-fos*, pS2, and Hsp 27 genes in order to inhibit ER-mediated transactivation of these genes (262, 291, 331).

There are several E2-induced genes that do not contain iDREs but are still inhibited by AhR agonists, and inhibitory AhR-ER α crosstalk may involve the proteasome-dependent degradation of ER α induced by both AhR and ER α agonists (Fig. 18) (264, 336). Wormke *et al.* showed that both E2 and TCDD activated proteasome-dependent degradation of ER α and AhR proteins respectively in MCF-7 and T47D cells (135). This study indicated that although TCDD was involved in the degradation of both ER α and AhR proteins, E2 was only involved in the proteasome degradation of ER α protein. These results indicated that proteasome degradation of ER α by TCDD may be a limiting factor that significantly contributes to AhR-mediated inhibition of E2 responses.

It has also been hypothesized that ligand bound AhR may compete with ER for coactivators leading to decreased E2-induced transactivation. Studies in this lab and others have revealed that AhR interacts with ER α coactivators such as

SRC-1 and ERAP140 (312, 337, 338) and the corepressor SMRT (312). These interactions may also contribute to inhibition of ER-mediated responses by AhR agonist.

Coimmunoprecipitation and GST-pulldown studies revealed that the AhR/Arnt complex interacted with ER and this interaction may contribute to AhR/ER crosstalk (339). Klinge *et al.* reported that the AhR interacted directly with ER α and inhibited E2-induced transactivation of genes with EREs such as pS2 and c-*fos*. However, Arnt did not interact with ER. Inhibition of gene expression may be caused by AhR sequestration of ER α and thereby inhibiting ER binding to DNA or interactions with other transcription factors including the basal transcription machinery. In contrast, studies by Ohtake *et al.* indicate that AhR/ER α interactions may not be inhibitory but estrogenic (340). This study reported that AhR/Arnt directly associated with ER α and ER β in MCF-7 cells treated with the AhR agonist 3-methylcholanthrene and that this complex was able to bind estrogen responsive gene promoters and recruit coactivators. This result was in agreement with other studies indicating that AhR agonists promote AhR/ER interactions (339). However, their observations that AhR agonists mediate estrogen-like responses have not been observed in research in this laboratory or by other investigators, and this is currently being reexamined in this laboratory (262, 268, 329-334).

AhR and Arnt have been shown to interact with the zinc finger domain of Sp1 through their basic HLH/PAS domains (341). These interactions may inhibit ER-mediated gene transactivation through competition with ER for Sp1. In genes where transactivation occurs through ER α /Sp1 interactions, the presence of the AhR and its interactions with Sp1 may not allow formation of the transcriptionally active ER α /Sp1 complex but a repressive AhR/Sp1 complex. Studies in this research project are focused on the cell context-dependent mechanisms of hormone-induced expression of E2F-1 and inhibition of this response by AhR agonists. The mechanisms of inhibitory AhR-ER α crosstalk will also be investigated and this will include studies on ER α /Sp1- AhR/Sp1 complex formation since hormone activation of E2F-1 is, in part, dependent on ER α /Sp1 (342, 343).

1.7 Research Objectives

Cell cycle progression and proliferation of tumors and normal tissues/cells are controlled by a constellation of factors that coordinately regulate one or more growth-dependent pathways (344-348). Many of the critical genes required for cell proliferation modulate progression through different phases of the cell cycle, and E2F family members play a critical role in G₁→S phase progression (349-352). Interactions of retinoblastoma (Rb) proteins with E2F suppresses transcription of genes which contain critical E2F binding sites; however, phosphorylation of Rb results in dissociation of the Rb-E2F complex and

subsequent upregulation of E2F-dependent genes (352-354). E2F-1 was the first E2F family member identified (355-357), and several studies have characterized E2F-1-dependent expression of genes required for cell proliferation, and this is related to the oncogenic activity of this transcription factor (349-352).

Although regulation of E2F-1-dependent transactivation is closely linked to Rb phosphorylation, E2F-1 expression is also modulated by many other nuclear transcription factors and coregulatory proteins (358-362). For example, p300/CBP-associated factor (P/CAF) acetylates E2F-1 and this enhances the transcriptional activity of E2F-1 in U20S cells (361).

Johnson and coworkers (358, 359) investigated cell cycle-dependent activity of constructs containing E2F-1 gene promoter fragments in REF-52 cells treated with serum and based on 5'-deletion analysis, the -204 to -122 region of the promoter was required for maximal responses. The E2F-1 binding sites in the proximal region of the promoter were primarily required for negative control of the E2F-1 promoter in G₀ and early G₁.

1.7.1 Objective 1

Research in this laboratory showed that 17 β -estradiol (E2) induced E2F-1 mRNA and protein levels in estrogen receptor (ER)-positive MCF-7 breast cancer cells, and the induction response was linked to cooperative ER α /Sp1/NFY interactions which involved three GC-rich (-169 to -111) and two

CCAAT (-122 to -54) binding sites (342). The downstream CCAAT sites bound NFYA and were required not only for hormone-dependent activation of E2F-1 but also for basal activity of the E2F-1 promoter. Studies in this laboratory have also used ER-positive ZR-75 breast cancer cells for investigating molecular mechanisms of hormone-induced transactivation of E2-responsive genes. MCF-7 and ZR-75 cells are both luminal epithelium cell lines that have been derived from human mammary tumors. Clinical studies have shown that women who have been diagnosed with ER α -positive breast cancer sometimes respond differently to chemotherapeutics due to the induction of different signaling pathways by growth regulators that lead to tumor formation (363). Other studies have also indicated that differences in protein expression in ER α -positive breast cancer cell lines can also determine the cells response to hormonal treatment. For example, de Cremoux *et al.* used two MCF-7 cell subtypes, the parental MCF-7 and a mutant MCF-7 cell lines, which had similar expression levels of ER α but different responses to hormonal therapy (364). There were differences in the expression of several genes including increased expression of multidrug resistance gene 1 and breast cancer antiestrogen resistance gene 1 in the mutant MCF-7 cell line, indicating that the hormonal regulation and responses to antiestrogens of some ER α -positive breast cancer cell lines may depend on the cell context. The first objective of this study is to compare hormonal activation of constructs containing the GC-rich and CCAAT motifs in the -173 to -54 region of

the E2F-1 gene promoter in ER α positive MCF-7 and ZR-75 cells. We hypothesized that although both cell lines share similar expression patterns of proteins such as ER α , AhR, and Arnt, some of the differences such as increased Sp1 protein expression in ZR-75 cells may lead to cell context-dependent differences in hormonal activation of E2F-1.

1.7.2 Objective 2

Studies in this laboratory have investigated an indirect inhibitory mechanism that involves the ligand-activated aryl hydrocarbon receptor (AhR) (334). The AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inhibits E2-induced expression of multiple genes/proteins including progesterone receptor, prolactin receptor, cell cycle proteins, cathepsin D, c-fos, pS2, and heat shock protein 27 in breast cancer cells (262, 291, 292, 296, 331, 332, 365, 366). The mechanisms of AhR-ER α crosstalk for some genes involve direct interaction of the AhR complex with inhibitory dioxin response elements (iDREs) in target gene promoters (165, 262, 291, 331, 332, 367) and also induction of proteasome-dependent degradation of ER α which becomes limiting in breast cancer cells (135, 368).

Troester *et al.* reported that MCF-7 and ZR-75 cell lines responded to chemotherapeutics better than basal epithelium cell lines that did not express ER α (369). This data is consistent with other studies indicating that ER α -positive cell lines respond to chemotherapeutics whereas ER α -negative cells do not respond

well. This study investigates the antiestrogenic effects of TCDD on E2-induced expression of E2F-1 in MCF-7, ZR-75 and Schneider SL-2 cells. We hypothesized that since E2-induced E2F-1 gene expression in a genomic and non-genomic manner, TCDD would inhibit E2-induced genomic and non-genomic mechanisms of E2F-1 gene expression.

CHAPTER II

METHODS AND MATERIALS

2.1 Chemicals and Cells

MCF-7, ZR-75, and Schneider SL-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were maintained in DME/F12 medium with phenol red and supplemented with 2.2 g/l sodium bicarbonate, 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO) in an air:carbon dioxide (95:5) atmosphere at 37°C. ZR-75 cells were maintained in RPMI medium supplemented with 10% FBS, 2.2 g/l sodium bicarbonate, 4.5 g/l glucose, 0.11 g/l pyruvic acid, 2.38 g/l HEPES, and 10 ml/l antibiotic-antimycotic solution in an air:carbon dioxide atmosphere at 37°C. For transient transfection studies, MCF-7 and ZR-75 cells were grown for 1 day in DME/F12 medium without phenol red and 2.5% FBS treated with dextran-coated charcoal, and for Western and Northern blot analysis, the same media without phenol red and FBS-free media was used for at least 48 h prior to treatment with E2 and other chemicals. SL-2 cells were cultured at room temperature in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS and 10

ml/l antibiotic-antimycotic. E2 was purchased from Sigma Chemical Co and TCDD was prepared in this lab and was >98% pure as determined by high-pressure liquid and gas chromatography. Dimethyl sulfoxide (DMSO) was used as a solvent for E2, SQ22536, 8-bromo-cAMP and MG132. SQ22536, 8-bromo-cAMP and MG132 were purchased from Calbiochem (La Jolla, CA). ER α , AhR, Sp1, Sp3, and NF-1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The NFYA antibody was purchased from Rockland, Inc. (Gilbertsville, PA). The PKA and phospho-PKA antibodies were purchased from Calbiochem. Luciferase and β -galactosidase enzyme assay systems are purchased from Promega Corp. (Madison, WI) and the cAMP enzyme immunoassay (EIA) system was purchased from Amersham Biosciences (Piscataway, NJ).

2.2 Cloning and Plasmids

The wild-type ER (hER) expression plasmid was provided by Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). Recombinant ER was obtained from Pan Vera Corp. (Madison, WI). The ER deletion mutants HE11, HE15, and HE19 were provided by Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France). The constructs pE2F-1a, pE2F-1b, and pE2F-1d were kindly provided by Dr. J.R. Nevins (Duke University, Durham, NC). The pE2F-1c construct were made by RT-PCR starting with the E2F-1

cDNA (forward primer: 5'-CCGCCATTGGCCGTACCGCCCC-3'; reverse primer: 5'-

GATCTTCCCGGCCACTTTTACGCGCCAAA-3') and inserted into pGL2 basic vector (Promega Corp.) at *Sac*I and *Bgl*II cloning sites. The remaining E2F-1 promoter constructs (pE2F-1e, pE2F-1f, pE2F-1g, pE2F-1h, pE2F-1h_{m1}, pE2F-1h_{m2}, pE2F-1h_{m3}, pE2F-1h_{m4}, pE2F-1h_{m5}, pE2F-1i, pE2F-1j, pE2F-1j_{m1}, pE2F-1j_{m2}, pE2F-1k, pE2F-1l) were made by inserting synthetic oligonucleotides into the pGL2 basic vector digested with *Sac*I and *Bgl*II enzymes at the cloning sites (Table 3). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Resulting plasmids were sequenced at the Gene Technology Laboratory (Texas A&M University, College Station, TX) to confirm appropriate insertion of the oligonucleotide inserts. The sequences of Sp1 (CCGCCCC) and CCAAT protein-binding sites in the E2F-1 promoter have been mutated into CCtttCC and atgcT, respectively, in all constructs containing mutations in these sites. The PKA expression plasmid was provided by Dr. R. Maurer (Oregon Health Sciences University, Portland, OR). The expression plasmids of wild type NFYA, NF-YB and mutant NFYA (Δ 4YA13_{m29}) and control plasmid (Δ 4YA13) were kindly provided by Dr. Roberto Mantovani (Universita di Milano, Milan, Italy). Gal4-luc was provided by Dr. M. Mayo (University of North Carolina, Chapel Hill, N.C). The pM-NFYA expression plasmid was made by PCR using primers (forward primer: 5'-GGA ATT CAT GGA GCA GTA TAC

GAC A-3'; reverse primer: 5'-GCT CTA GAT TAG GAA ACT CGG ATG A-3') to amplify full length NFYA using the NFYA expression plasmid as a template. The amplified products were cloned into the pM vector (Clontech, Palo Alto, CA) between EcoRI and XbaI cloning sites. The pPAC and pPAC/Sp1 expression vectors were provided by Dr. Robert Tjian (University of California-Berkeley, Berkeley, CA) and the pPAC/hER α expression plasmid was made in this laboratory by releasing the hER α coding sequence from the hER α expression plasmid and cloning it into the XhoI site of pPAC.

Table 3. *Wild type E2F-1 and deletion and mutation constructs^a.*

| Construct | Oligonucleotide Sequence |
|-------------------------------|---|
| pE2F-1b -242/+77 | cgggctgggggagtcagaccgcgcctgtaccatccggacaaagcctgcgcgcgccccgcc cgccattggcgtaccgccccgcgcgcgcgccccatctcgccctcgccgcccgggtccggcggttaa gccaataggaaccgcgcgcgtgttccgtcacggccggggcagccaattgtggcgcgctcgccggct cgtggctcttcgcggcaaaaaggattggcgcgtaaaagtggccgggacttgcaggcagcgccggcc ggggcgagcgggatcgagccctcgccgagcctgcgcgcctg |
| pE2F-1c -177/+10 | cccgccattggcgtaccgccccgcgcgcgcgccccatctcgccctcgccgcccgggtccggcggtta aagccaataggaaccgcgcgcgtgttccgtcacggccggggcagccaattgtggcgcgctcgccg gctcgtggctcttcgcggcaaaaaggattggcgcgtaaaagtggccg |
| pE2F-1d -122/+77 | gtccggcgcgtaaaagccaataggaaccgcgcgcgtgttccgtcacggccggggcagccaattgtg cgccgctcgccggctcgtggctcttcgcggcaaaaaggattggcgcgtaaaagtggccgggacttgc aggcagcgcgccggggggcgagcgggatcgagccctcgccgagcctgcgcgcctg |
| pE2F-1e -242/-122 | cgggctgggggagtcagaccgcgcctgtaccatccggacaaagcctgcgcgcgccccgcc cgccattggcgtaccgccccgcgcgcgcgccccatctcgccctcgccgccc |
| pE2F-1f -242/-122 -20/0 | cgggctgggggagtcagaccgcgcctgtaccatccggacaaagcctgcgcgcgccccgcc cgccattggcgtaccgccccgcgcgcgcgccccatctcgccctcgccgcccgaaggattggcg gtaa |
| pE2F-1g -173/-54 | ccattggcgtaccgccccgcgcgcgcgccccatctcgccctcgccgcccgggtccggcggttaaagc caataggaaccgcgcgcgtgttccgtcacggccggggcagccaattgtg |
| pE2F-1h -169/-54 | tggcgtaccgccccgcgcgcgcgccccatctcgccctcgccgcccgggtccggcggttaaagccaat aggaaccgcgcgcgtgttccgtcacggccggggcagccaattgtg |
| pE2F-1 hm1 -169/-54 | tggcgtacccttcgcgcgcgcgccccatctcgccctcgccgcccgggtccggcggttaaagccaata ggaaccgcgcgcgtgttccgtcacggccggggcagccaattgtg |

2.3 Transient Transfection and Luciferase Assays

MCF-7 and ZR-75 cells were seeded in 12 well Falcon plates in DME/F12 medium supplemented with 2.5% dextran-coated charcoal FBS and grown until they were 70% confluent. Plasmids (500 ng) were transiently cotransfected with the ER expression plasmid (500 ng) using the calcium phosphate method. Cells were incubated for 4 to 6 h and then shocked with 25% glycerol in phosphate-buffered saline (PBS) and treated with DMSO, 10 nM E2, 10 nM TCDD, and TCDD plus E2 and kinase inhibitors and activators or their combinations in DMSO for 44 to 48 h. SL-2 cells were transfected with the E2F-1 promoter construct (500 ng), and cotransfected with pPAC plasmids using the calcium phosphate method. The SL-2 cells were incubated for 16 h and treated with DMSO, 10 nM E2, 10 nM TCDD, and TCDD plus E2 for 44 to 48 h. The cells were harvested in cell lysis buffer (Promega Corp.) Cell lysates were prepared by freeze-thawing followed by centrifugation at 14,000 X *g* for 1 min. Luciferase activity was then determined in a luminometer (Packard Instruments Co. Meriden, CT) with a luciferase assay kit (Promega Corp.) and normalized to β -galactosidase enzyme activity obtained after transfection with a β -galactosidase-lacZ plasmid (500 ng). The pcDNA3.1/His/LacZ control vector containing the gene for b-galactosidase and a CMV promoter was from Invitrogen (Carlsbad, CA). The experiments for each treatment group were carried out at least in triplicate.

2.4 Northern Blot Analysis

MCF-7 and ZR-75 cells were seeded and grown as described above and treated with DMSO, 10 nM E2, 10 nM TCDD, and TCDD plus E2. RNA was extracted using an RNA extraction kit from Tel-Test (Friendswood, TX). Total RNA (25 µg) was separated on a 1.2% agarose/1 M formaldehyde gel and transferred onto nylon membrane. The membrane was then exposed to UV light for 5 min to crosslink RNA to the membrane and then baked at 80°C for 2 h. The membrane was prehybridized in a solution containing 0.1% BSA, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone, 10% dextran sulfate, 1% SDS, and 5x SSPE (0.75 M sodium chloride, 50 mM NaH₂PO₄, 5 mM EDTA) for 18 to 24 h at 60°C and hybridized in the same buffer for 24 h with the [³²P]-labeled DNA probe (10⁶ cpm/ml). The E2F-1 cDNA probes (21) was labeled with [γ -³²P]dCTP using the random primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, IN). The resulting blots were visualized and quantitated using a Molecular Dynamics, Inc. Storm 860 instrument (Molecular Dynamics, Inc., Sunnyvale, CA). β -Actin mRNA was used as an internal control to standardize E2F-1 mRNA levels.

2.5 Preparation of Nuclear Extracts

Nuclear extracts were prepared from cells treated with DMSO or E2. Harvested cells were washed twice in 30 ml of HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, pH 7.6). The pellet was then incubated for 10 min in 1 ml of HED buffer (HEGD without the glycerol). Cells

were then transferred to a 2 ml homogenizing tube and homogenized using a Teflon/pestle drill apparatus. The homogenate was transferred to a centrifuge tube, centrifuged at 4000 X *g* for 10 min, washed twice with HEGD, and finally resuspended in 2 ml of HEGD containing 0.5 M sodium chloride and allowed to incubate at 4°C for 1 h.

2.6 Gel Mobility Shift Assay with Nuclear Extracts

Synthetic oligonucleotides were synthesized, purified, annealed and labeled at the 5'-end using T4-polynucleotide kinase and [γ -³²P] ATP. DNA binding was measured using a gel retardation assay. Nuclear extracts were incubated in HEGD with 1 μ g poly[d(I-C)] and 1 mM ZnCl for 10 min on ice to bind nonspecific DNA-binding proteins. Then 200-fold excess of unlabeled wild type or mutant oligonucleotide competitors for the competition experiments were incubated with the nuclear extracts for 5 min on ice. The mixture was then incubated for 15 min at 20°C with 0.1pmol/ μ l [³²P]-labeled DNA probe. Antibodies were added for an additional 15 min for the supershift reactions. The reaction mixture was then loaded onto a 5% polyacrylamide gel and electrophoresed at 150 V for 2.5 h in 0.9 M tris-borate and 2 mM EDTA, pH 8.0. The gel was dried and protein-DNA complexes were visualized using a Molecular Dynamics, Inc. Storm 860 instrument (Molecular Dynamics, Inc., Sunnyvale, CA)

2.7 Western Blots

MCF-7 and ZR-75 cells were grown and seeded as described above. Cells were synchronized in FBS-free DME-F12 media for 48 h and treated with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for various time points. For experiments where MG132 was used, cells were pretreated with 10 μ M MG132 and DMSO for 30 min and then treated with DMSO, E2, TCDD, and E2 plus TCDD for various time points. The cells were then lysed in ice-cold lysis buffer (50 mM HEPES [pH 7.5], 500 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% (v/v) glycerol, and 1% (v/v) Triton X) supplemented with protease inhibitor cocktail (Sigma). Equal amounts of protein from each treatment group were boiled in 1X Laemmli Buffer (50 mM Tris-HCL, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 175 mM β -mercaptoethanol) and separated by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in Blotto (5% milk, Tris-buffered saline [10 mM Tris-HCL, pH 8.0, 150 mM NaCl] and 0.05% Tween 20) and probed with primary antibodies (ER α , AhR, and Sp1). Following incubation with peroxidase-conjugated secondary antibodies, immunoglobulins were visualized using the Western Lightning ECL detection system (PerkinElmer Life Sciences, Inc., Boston, MA). Quantitation was performed using a Sharp JX-330 scanner (Sharp Electronics, Mahwah, NJ) and Zero-D Scanalytics software (Scanalytics Corp. Sunnyvale, CA).

2.8 cAMP Enzyme Immunoassay (EIA)

ZR-75 cells were seeded in 2.5% FBS DME/F12 medium overnight. The cells were then treated with the various compounds for 30, 60, and 120 min. The cAMP EIA system (Amersham) was used to determine cAMP level according to the supplier's instructions. The calculations for cAMP levels (pmol/mg protein) were based on a standard curve for each experiment.

2.9 Statistical Analysis

The statistical difference between different groups was determined by ANOVA and Student's *t* test. The data are expressed as means \pm standard errors or standard deviations. At least three determinations were carried out for each data point in the transfection and mRNA studies.

CHAPTER III

RESULTS*

3.1 Cell Context-dependent Differences in the Induction of E2F-1 Gene

Expression by 17 β -estradiol in MCF-7 and ZR-75 Cells

3.1.1 Comparative Analysis of the E2F-1 Promoter in MCF-7 and ZR-75 Cells

Previous studies show that E2 induces E2F-1 gene expression and transactivation in MCF-7 cells. In order to determine if E2 induced E2F-1 gene expression and transactivation, mRNA, protein and transactivation studies were conducted in order to compare E2F-1 activation in MCF-7 and ZR-75 cells. ZR-75 cells were treated with E2 at different time points and similar results were obtained in ZR-75 cells where a > 2.5-fold increase in E2F-1 mRNA levels was observed after treatment with 10 nM E2 for 6 h (Fig. 19). Previous studies showed that the -173 to -54 region of the E2F-1 gene promoter exhibited high basal activity and, in transient transfection assays in MCF-7 cells, E2 induced reporter gene (luciferase) activity (342). The results in Fig. 20A show that E2F-1h (containing the -169 to -54 E2F-1 promoter insert) was E2- responsive

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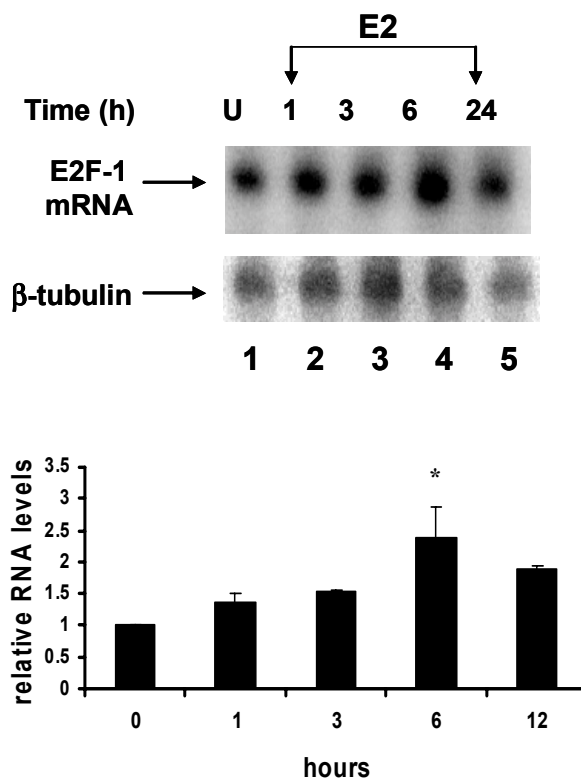


Fig. 19. Hormone-induced transactivation of E2F-1 mRNA in breast cancer cells. ZR-75 cells were treated with 10 nM E2 for 1, 3, 6 and 24 h, and E2F-1 and β -tubulin mRNA levels were determined by Northern blot analysis as described in the Materials and Methods. E2F-1 mRNA levels in untreated cells (U) are also indicated, and significant ($p < 0.05$) induction is indicated with an asterisk. Results are expressed as means \pm SE for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments.

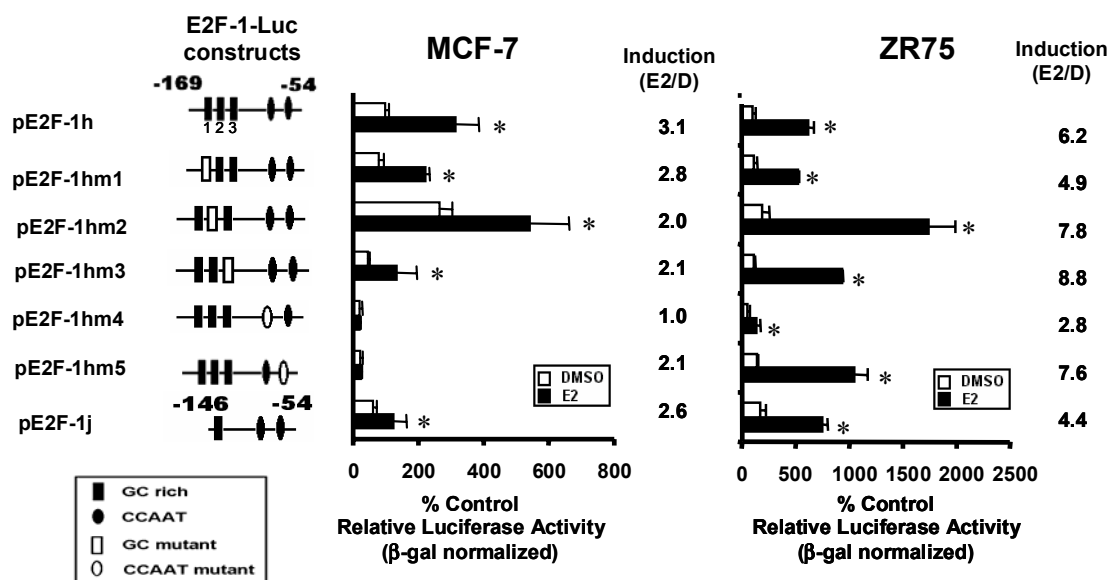


Fig. 20. Deletion and mutation analysis of E2F-1 promoter constructs in MCF-7 (left) and ZR-75 (right) cells. Constructs containing wild-type or mutant E2F-1 gene promoter inserts from the hormone-responsive -169 to -54 region were cotransfected with hER and B-galactosidase into MCF-7 or ZR-75 breast cancer cells, treated with DMSO (solvent control) or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by E2 is indicated with an asterisk, and fold induction by E2 is also given. Results are expressed as means \pm SE for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments for pE2F-1h.

in both MCF-7 and ZR-75 cells. Basal and hormone-induced activity of a series of GC- rich and CCAAT mutants (pE2F-1h_{m1-m5}) exhibited both similarities and differences in the two cell lines. Compared to pE2F-1h basal activity among the GC-rich mutants (pE2F-1h_{m1}, pE2F-1h_{m2} and pE2F-1h_{m3}) varied by > 4-fold in MCF-7 cells, and this was primarily due to site 2 where mutation significantly increased basal activity; mutation of site 3 decreased activity. These differences in basal activity were only 2-fold in ZR-75 cells and this was also due to the GC-rich site 2 suggesting that this motif may preferentially bind inhibitory factors such as Sp3. Mutation of the upstream (pE2F-1h_{m4}) or downstream (pE2F-1h_{m5}) CCAAT sites significantly decreased basal activity in MCF-7 cells; in contrast, loss of basal activity in ZR-75 cells was only observed for the former construct indicating important differences between contributions of the two CCAAT sites in determining basal activity of this region of the E2F-1 gene promoter. The importance of the upstream CCAAT motif was also observed in hormone-responsiveness of this series of mutants (Fig. 20). The only major difference in the effects of E2 on this series of constructs was that mutation of the upstream or downstream CCAAT elements resulted in loss of E2-responsiveness in MCF-7 cells transfected with pE2F-1h_{m4} or pE2F-1h_{m5}, whereas the latter (but not the former) construct was hormone-responsive in ZR-75 cells. The results observed in MCF-7 cells were consistent with cooperative

ligand (E2)-dependent formation of an ER α /Sp1/NFYA complex in which both *cis*-elements are required for transactivation (342). However, if this complex is functional in ZR-75 cells, then at least the downstream (3') CCAAT site is not required. The results in Fig. 21 confirm that hormone-inducibility in MCF-7 cells transfected with pE2F-1j is lost with constructs in which the GC-rich site is deleted (pE2F-1j_{m1}) or the CCAAT sites are mutated (pE2F-1j_{m2}). In contrast, both wild-type and mutant pE2F-1j constructs were hormone-responsive in ZR-75 cells demonstrating that the CCAAT sites alone were hormone-responsive. Fig. 22 investigates the activity of the three GC-rich sites alone in the -169 to -111 region of the E2F-1 gene (pE2F-1k) or in combination with the downstream (pE2F-1h), upstream (pE2F-1l) or both upstream/downstream (pE2F-1g) CCAAT motifs. Results obtained in MCF-7 cells clearly demonstrated that hormone-responsiveness required both the GC-rich and downstream CCAAT motifs, whereas the GC-rich sites alone were sufficient for E2-induced transactivation in ZR-75 cells.

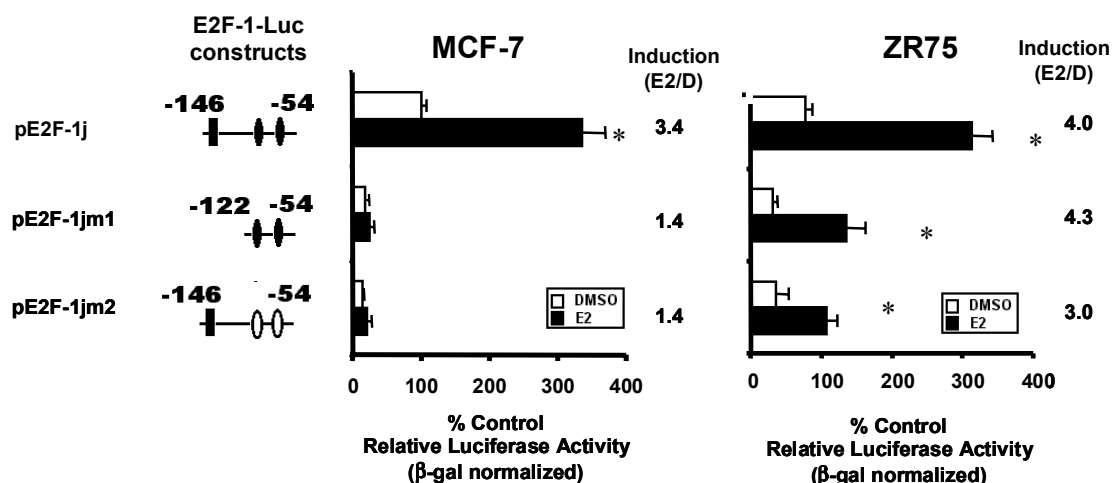


Fig. 21. Deletion and mutation analysis of E2F-1 minimal promoter region in MCF-7 (left) and ZR-75 (right) cells. Constructs containing wild-type or mutant E2F-1 gene promoter inserts from the hormone-responsive -146 to -54 region were cotransfected with hER and B-galactosidase into MCF-7 or ZR-75 breast cancer cells, treated with DMSO (solvent control) or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by E2 is indicated with an asterisk, and fold induction by E2 is also given. Results are expressed as means \pm SE for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments in cells transfected with pE2F-1j.

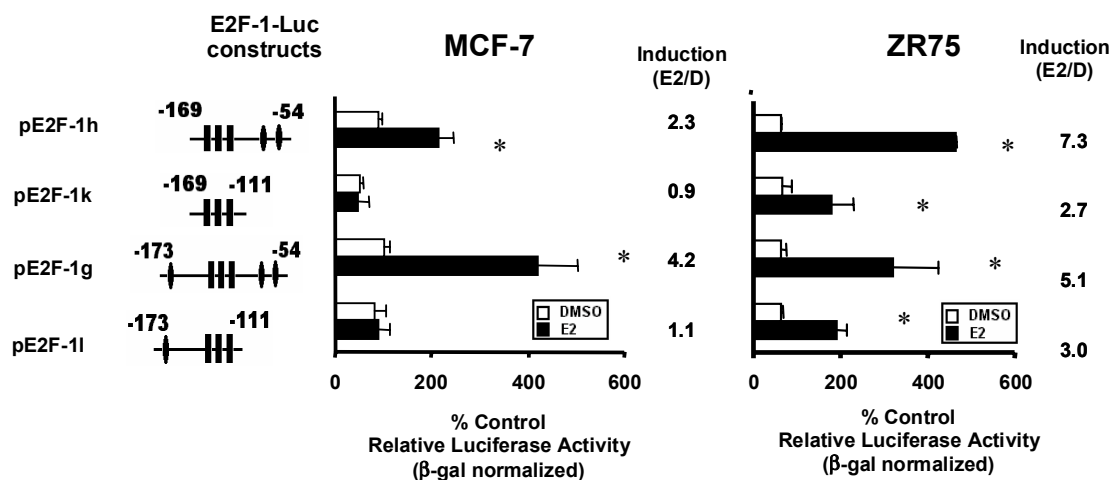


Fig. 22. Deletion analysis of the CCAAT sites on the E2F-1 promoter constructs in MCF-7 (left) and ZR-75 (right) cells. Constructs containing wild-type or mutant E2F-1 gene promoter inserts from the hormone-responsive -173 to -54 region were cotransfected with hER and β -galactosidase into MCF-7 or ZR-75 breast cancer cells, treated with DMSO (solvent control) or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by E2 is indicated with an asterisk, and fold induction by E2 is also given. Results are expressed as means \pm SE for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments in cells transfected with pE2F-1h.

3.1.2 Comparative Activation of E2F-1 Promoter Constructs by Wild-type and Variant ER α Expression Plasmids

Previous studies have demonstrated that E2 induced reporter gene activity in MCF-7 and ZR-75 cells transfected with an E2-responsive GC-rich constructs and wild ER α or a DBD deletion mutant (HE11) (141, 172-174, 176, 370, 371). This mutant was also shown to be expressed in HeLa cells by Western blot analysis and its binding to the estrogen response element was very weak compared to wildtype ER α (372, 373). MCF-7 and ZR-75 cells were cotransfected with E2F-1 promoter constructs, wild type ER α , and variant ER α mutant expression plasmids in order to determine the role of the ER α functional domains in E2-induced E2F-1 gene transactivation. The results in Fig. 23 show that E2 induced luciferase activity in MCF-7 cells transfected with pE2F-1h or pE2F-1j and wild-type ER α but not HE11. These results are consistent with the observation that hormone-responsiveness in MCF-7 cells requires both GC-rich and CCAAT motifs to form an ER α /Sp1/NFYA complex which was not activated in cells transfected with HE11 (342). Thus, formation of the ER α /Sp1/NFY complex in MCF-7 cells does not allow direct activation of ER α /Sp1 on GC-rich sites alone. Both ER α and HE11 activated pE2F-1h, pE2F-1j, and pE2F-1j_{m2} in ZR-75 cells, whereas pE2F-1j_{m1} containing only CCAAT sites was not induced by E2. This was consistent with the hormone-responsiveness of the GC-rich

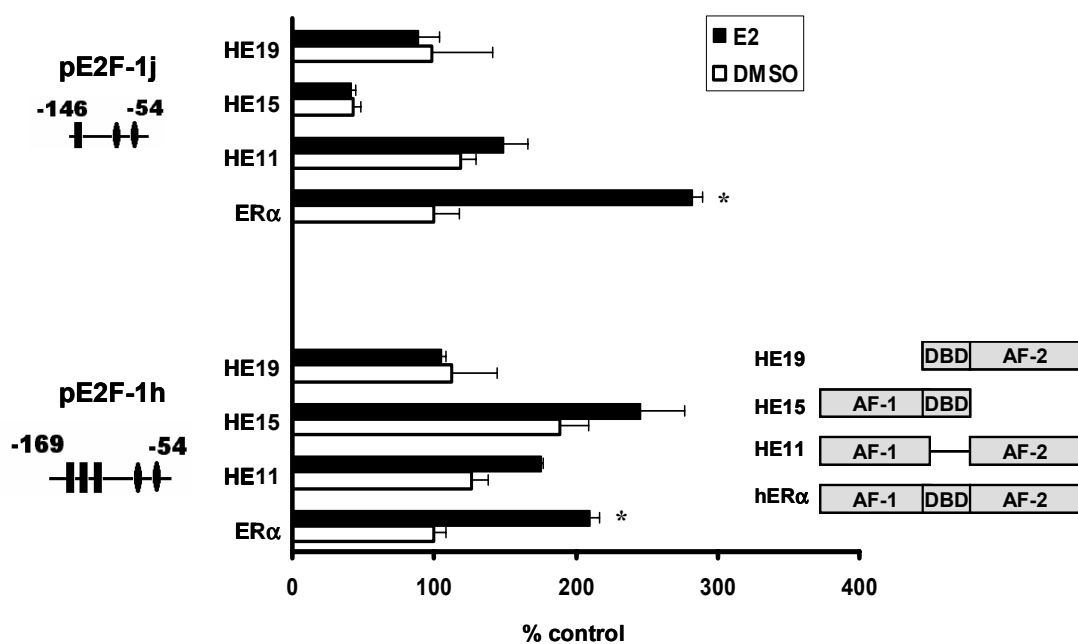


Fig. 23. Comparative activation of E2F-1 constructs by wild-type and variant ERα constructs in MCF-7 breast cancer cells. MCF-7 cells were cotransfected with constructs containing E2F-1 promoter inserts and ERα or HE11, HE15 and HE19 and treated with DMSO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated with an asterisk, and results are expressed as means \pm SD for three replicate determinations for each treatment group.

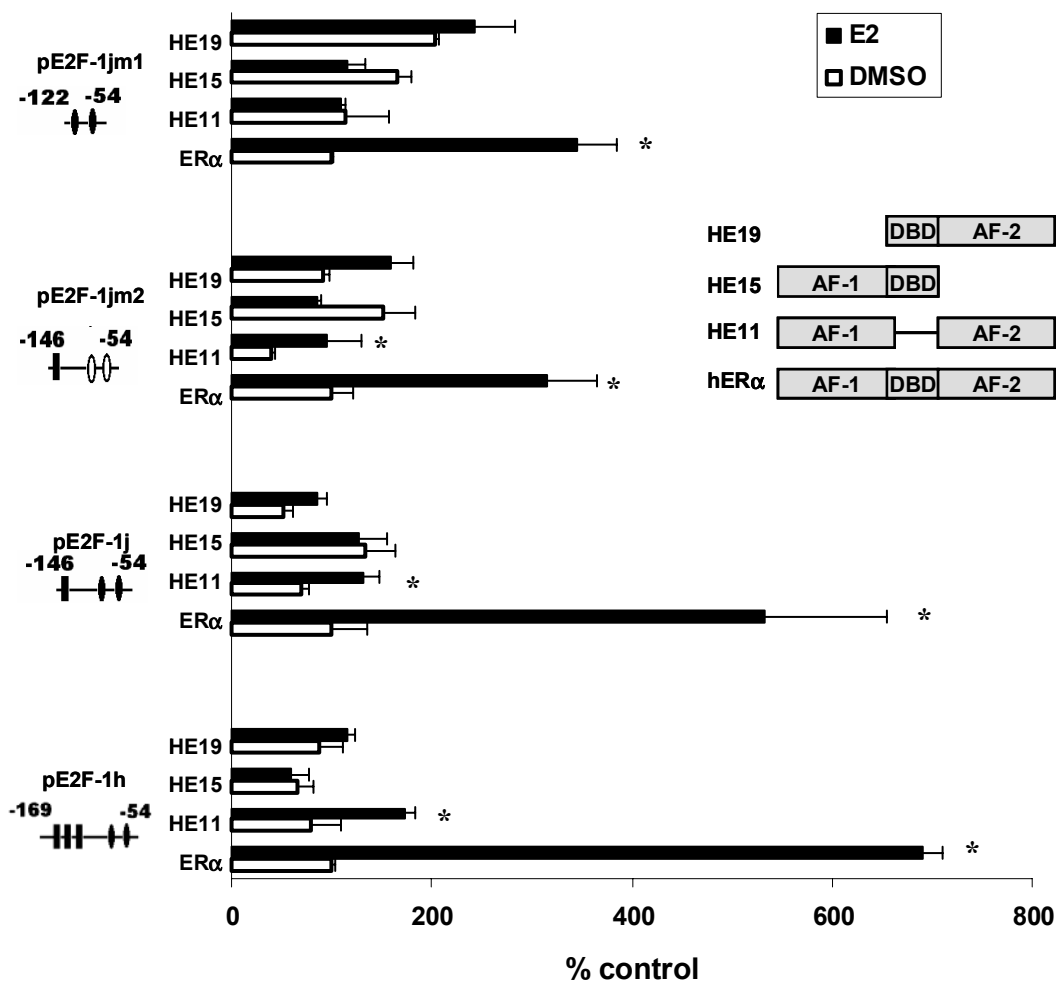


Fig. 24. Hormonal activation of ER α mutant constructs in ZR-75 breast cancer cells. ZR-75 cells were cotransfected with constructs containing E2F-1 promoter inserts and ER α or HE11, HE15 and HE19 and treated with DMSO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated with an asterisk, and results are expressed as means \pm SD for three replicate determinations for each treatment group.

sites alone in ZR-75 cells (141, 172-174, 176, 370, 371) and contrasted to the results obtained in MCF-7 cells (Fig. 24). ER α mutants containing N-terminal (HE19) and C-terminal (HE15) deletions were not hormone-responsive in either cell line (Fig. 23 and Fig. 24).

3.1.3 Role of NFYA in E2-induced Transactivation of E2F-1 in MCF-7 and ZR-75 Cells

CCAAT sites have been shown to bind the NF-Y heterotrimer that is comprised of NF-YA, NF-YB, and NF-YC. NF-YB and NF-YC are responsible for inducing formation of the NF-Y complex and interacting with other coregulators, while NF-YA is responsible for binding to the DNA CCAAT motifs (374, 375). In order to confirm the role of NF-Y in E2-induced E2F-1 transactivation, MCF-7 and ZR-75 cells were cotransfected with E2F-1 promoter constructs, ER α , and NF-Y expression plasmids. E2 induces luciferase activity in MCF-7 and ZR-75 cells transfected with pE2F-1j (Figs. 25A and 25B), and cotransfection with NFYA, NF-YB or 4YA13 which does not exhibit dominant negative activity did not affect the induction response. In contrast, cotransfection with the dominant negative 4YA13_{m29} expression plasmid for NFYA blocks hormone activation in MCF-7 cells and this is consistent with the reported hormone-dependent ER α /Sp1/NFYA-mediated transactivation which requires both GC-rich and CCAAT sites (342). In contrast, NFYA dominant negative decreases the basal levels as well as the E2 response,

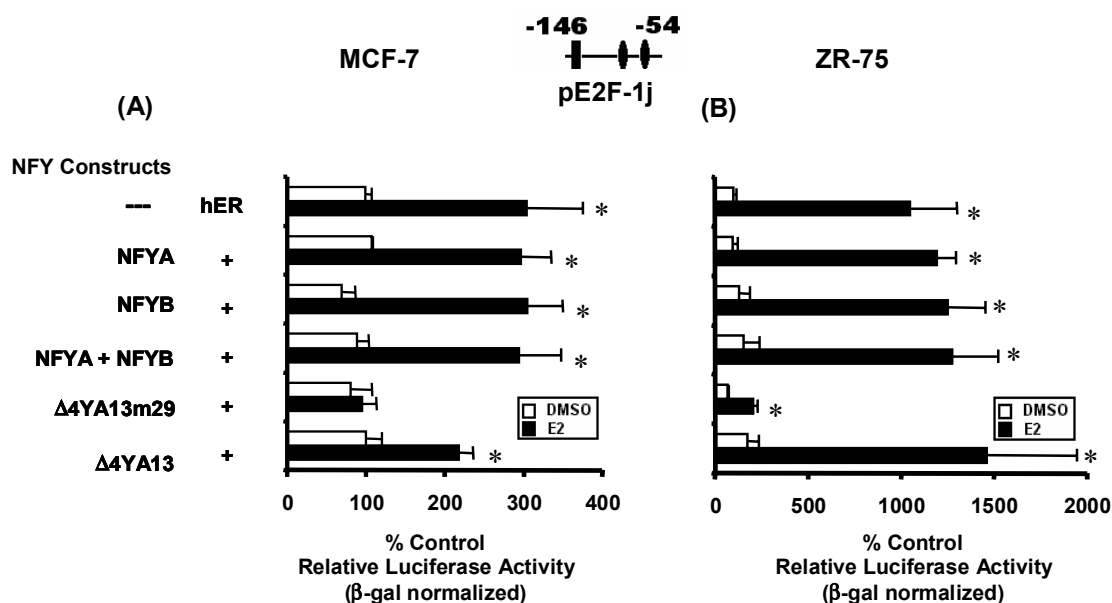


Fig. 25. Effects of NFYA and transactivation. Transfection of pE2F-1j in MCF-7 (A) and ZR-75 (B) cells. Transfection of pE2F-1j in MCF-7 (A) and ZR-75 (B) cells. Cells were transfected with pE2F-1j and expression plasmids for hER, NFYA, NF-YB, NFYA + NF-YB, 4YA13_{m29} or 4YA13, treated with DMSO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk and results are expressed as means \pm SD for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments in cells transfected pE2F-1j.

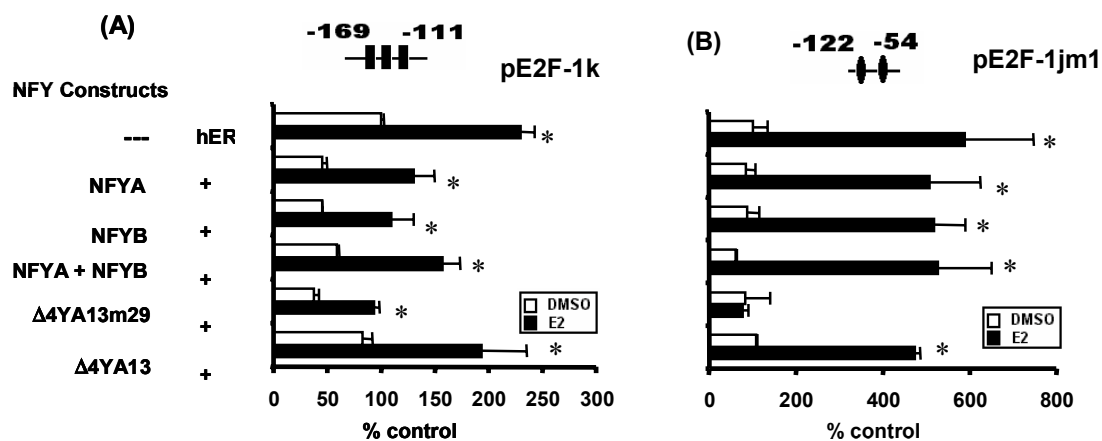


Fig. 26. Role of NF-YA on transactivation in cells transfected with pE2F-1k and pE2F-1jm1. Transfection of ZR-75 cells with pE2F-1k [A] or pE2F-1jm1 [B]. Cells were transfected with pE2F-1k or pE2F-1jm1 and expression plasmids for hER, NF-YA, NF-YB, NFYA + NF-YB, 4YA13_{m29} or 4YA13, treated with DMSO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk and results are expressed as means \pm SD for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments in cells transfected pE2F-1k [A] and pE2F-1jm1 [D].

it does not eliminate hormone-induced transactivation in ZR-75 cells transfected with pE2F-1j (Fig. 25B) and this is consistent with E2-responsiveness of the GC-rich sites alone in ZR-75 cells (Fig. 26A). The decrease in basal levels is most likely due to other factors binding to the CCAAT sites and either recruiting corepressors and/or not allowing interaction with the basal transcription machinery. The effects of NFYA, NF-YB, 4YA13 and 4YA13_{m29} expression plasmids on hormone-inducibility of pE2F-1k (Figure 26A) and pE2F-1j_{m1} (Fig. 26B) were also investigated in ZR-75 cells. The results indicate that hormone inducibility in cells transfected with the GC-rich construct (pE2F-1k) were unaffected by any of the expression plasmids, whereas the dominant negative NFYA expression plasmid blocked induction of pE2F-1j_{m1} suggesting that NFYA plays a key role in direct hormone activation through the CCAAT motifs in ZR-75 cells.

The results in Fig. 26A illustrate that dominant negative NFYA (4YA13_{m29}) and NFYA expression plasmids do not affect ER α /Sp1-mediated activation of pE2F-1k which contains three GC-rich Sp1 binding sites. In contrast, Farsetti and coworkers showed that NFYA physically interacts with ER α and inhibits ER α action in NIH3T3 and HepG2 cells transfected with an ERE construct. Moreover, this response was also observed using a form of NFYA that does not bind DNA (376). Our results show that NFYA does not affect ER α /Sp1-mediated activation of GC-rich constructs (Fig. 26A) and cotransfection with

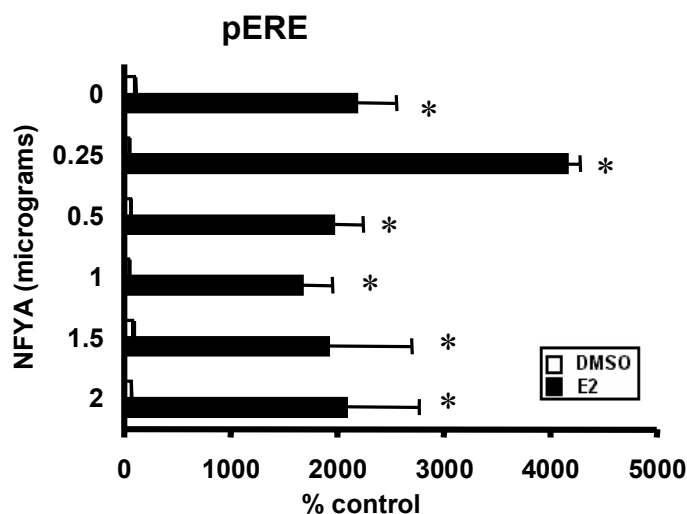


Fig. 27. Effects of NFYA on hormonal activation of pERE. ZR-75 cells were transfected with pERE and different amounts (0.25 – 2.0 μ g) of NFYA expression plasmid, treated with DMSO or 10 nM E2, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk and results are expressed as means \pm SD for three replicate determinations for each treatment group. The 100% control values were the DMSO treatments in cells transfected pERE alone.

ER α activates constructs containing only CCAAT motifs (Figs. 21,24, and 26). Therefore, we investigated potential inhibitory interactions of NFYA on ER α in ZR-75 cells transfected with pERE₃ (Fig. 27). These results show that NFYA does not inhibit ER α -mediated transactivation in ZR-75 cells as previously reported in other cell lines (376) demonstrating that inhibitory NFYA-ER α interactions are dependent on cell context.

3.1.4 Interaction of NFY and Sp Proteins with the E2F-1 Promoters

E2-treated nuclear extracts from MCF-7 and ZR-75 cells were used in order to confirm that Sp1 and NF-YA bound the GC-rich and CCAAT motifs, respectively. Interactions of nuclear extracts from untreated and E2-treated MCF-7 and ZR-75 cells with [³²P]-122/-54 oligonucleotide were investigated in gel mobility shift assays (Fig. 28). A major protein-DNA retarded band was observed with both extracts in MCF-7 (lanes 2 and 3) and ZR-75 (lanes 12 and 13) cells, and it was apparent that treatment with E2 did not affect retarded band intensities. In competitive DNA-binding experiments with unlabeled -122/-54 or consensus NF-Y oligonucleotides, there were significant decreases in retarded band intensities using extracts from MCF-7 (lanes 4 and 5) or ZR-75 (lanes 14 and 15) cells. In contrast, competition with unlabeled consensus NF-1, Sp1 (GC-rich) or ERE oligonucleotides had minimal effects on retarded band intensities (lanes 6 - 8 and 16 - 18). NF-1 antibodies did not supershift the retarded band in MCF-7 (lane 10) or ZR-75 (lane 20) cells. The results

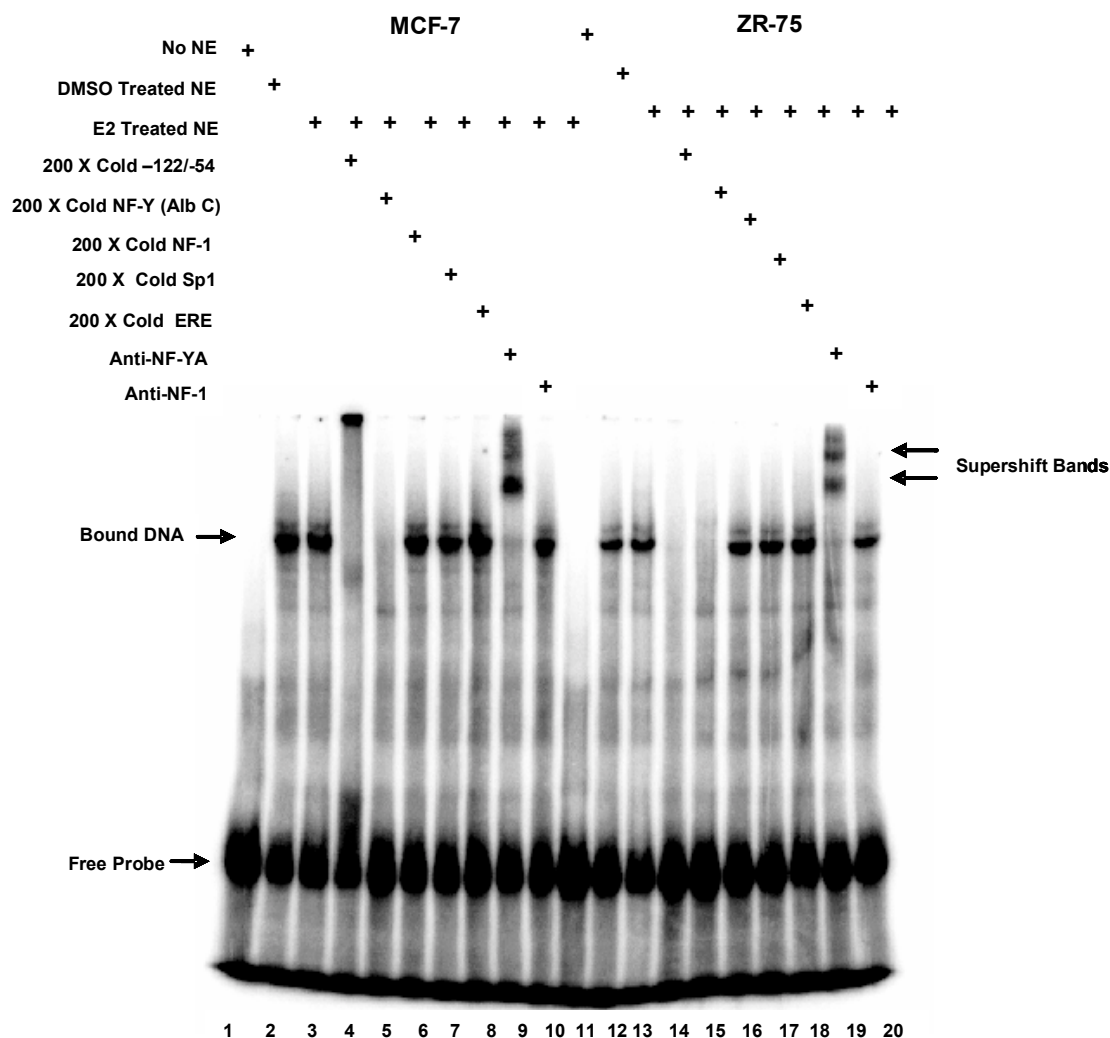


Fig. 28. Gel electrophoretic mobility shift assays for -122/-54 binding. Nuclear extracts from MCF-7 or ZR-75 cells were incubated with [32 P]-122/-54 and various unlabeled oligonucleotides or antibodies (NFYA or NF-1) and analyzed by gel mobility shift assays as described in the Materials and Methods. Results were observed in duplicate experiments.

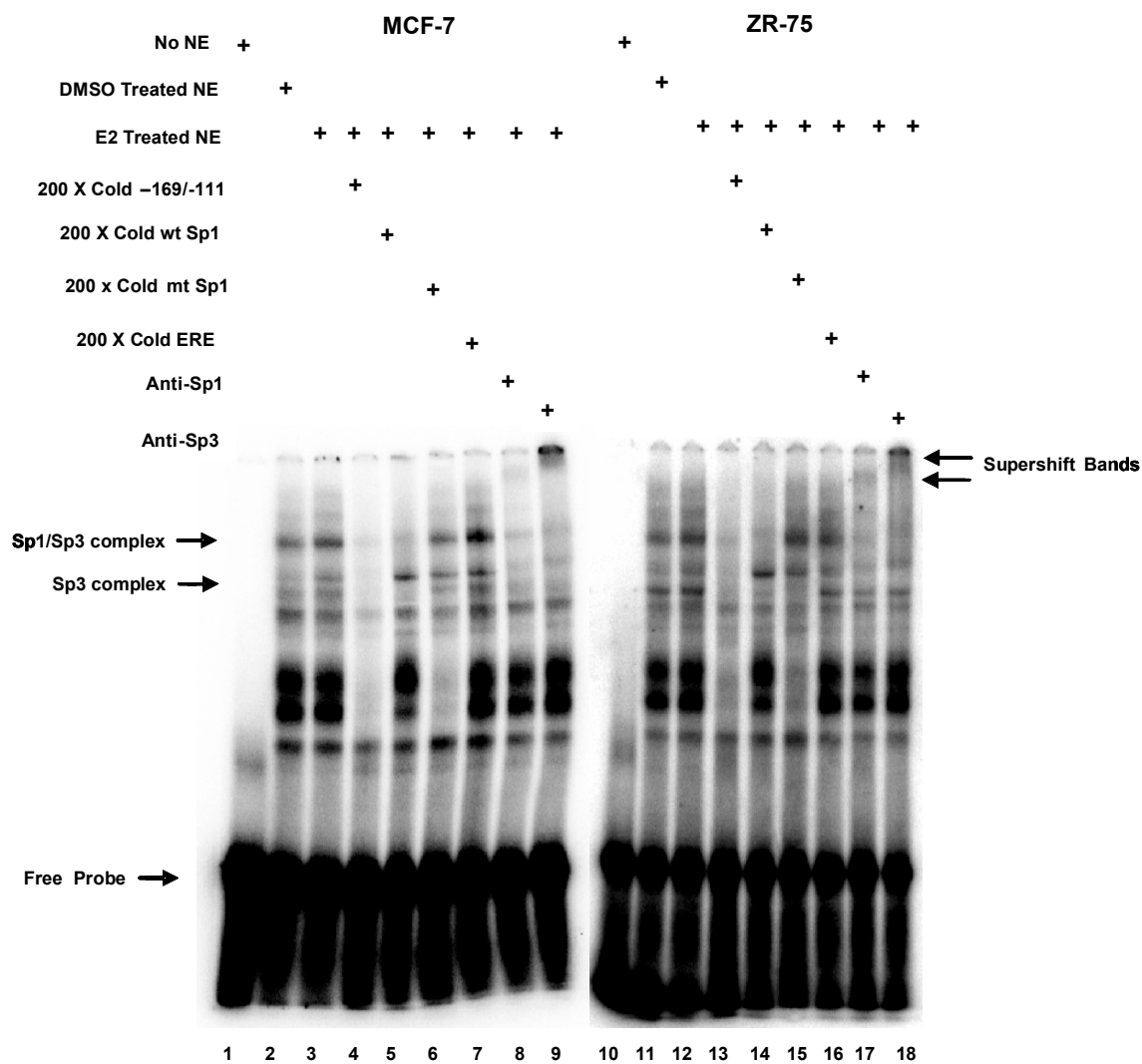


Fig. 29. Gel electrophoretic mobility shift assays for -169/-111 binding. Nuclear extracts from MCF-7 or ZR-75 cells were incubated with [32 P]-169/-111 and unlabeled nucleotides or antibodies (Sp1 or Sp3), and analyzed by gel mobility shift assays as described in the Materials and Methods. Results in these assays were observed in duplicate experiments and specifically bound complexes and supershifted bands are indicated.

demonstrate that despite the differences in the requirements for the CCAAT sites for hormonal activation of constructs derived from the E2F-1 gene promoter, the pattern of retarded bands observed using [32 P]-122/-54 was similar for nuclear extracts from MCF-7 or ZR-75 cells. Moreover, the results also indicate that hormonal activation of CCAAT sites in ZR-75 cells was not due to enhanced binding of E2-induced nuclear extracts to [32 P]-122/-54.

Interaction of nuclear extracts from both cell lines with the GC-rich -169 to -111 region of the E2F-1 promoter were also comparable (Fig. 29). Extracts from both cell lines formed multiple bands including a higher molecular weight complex previously associated with DNA interactions with Sp1 proteins (342) (bound DNA) (lanes 2, 3, 11 and 12). Intensity of this "bound band" was decreased after competition with unlabeled -169/-111 and consensus Sp1 oligonucleotides (lanes 4, 5, 13 and 14) but not after competition with unlabeled mutant Sp or ER oligonucleotides (lanes 6, 7, 15 and 16). Coincubation with Sp1 or Sp3 antibodies gave supershifted bands with extracts from MCF-7 (lanes 8 and 9) or ZR-75 (lanes 17 and 18) cells. Thus, extracts from both cell lines form Sp1 and Sp3 complexes with the GC-rich -169 to -111 sequence from the E2F-1 gene promoter. In this study and previous reports (172, 342), we did not observe ER α associated with Sp1 or NFYA in gel mobility shift assays, even though ER α physically interacts with both proteins. However, ER α enhances Sp1-DNA complex formation (on-rate) and stabilized the NFYA-DNA complex

which was also enhanced by Sp1 (342). The failure to observe ternary protein-protein-DNA complexes in gel shift assays has also been reported for other interacting transcription factors. For example, cyclin D1, sterol regulatory element binding protein, and the human T cell lymphotropic virus type 1 Tax protein enhance binding of ER, Sp1 and c-jun to their cognate response elements, respectively, but do not form a supershifted complex (377-379).

3.1.5 Hormonal Activation of CCAAT Sites through Non-genomic Pathways

Since there are no GC-rich sites or estrogen response elements present in the -122 to -54 region of the E2F-1 promoter, ER α -mediated transactivation is not induced in a genomic ER α /Sp1 or ER α /ERE dependent manner. Therefore, there may be an alternative non-genomic mechanism for E2 induced E2F-1 gene expression. E2 has been shown to activate the cAMP/PKA pathway in cancer cell lines (218, 380-383) and the cAMP/PKA pathway has also been shown to activate the NF-Y complex (384). The next series of experiments was conducted in order to determine if the E2F-1 CCAAT sites were activated through cAMP/PKA phosphorylation of the NF-Y complex. The adenylyl cyclase/cAMP inhibitor SQ22536 (400 μ M) blocked E2-induced luciferase activity in cells transfected with pE2F-1_{j_m1} (Fig. 30), and this inhibitor did not affect cell viability. Previous studies showed that SQ22536 inhibited cAMP/PKA activation of a cAMP response element in ZR-75 cells (177), suggesting that this

pathway may be required for activation of the CCAAT sites. This observation was consistent with the known activation of this pathway by E2 (175, 186, 385-387) as well as a recent report showing that cAMP inducers activate tissue inhibitor of metalloproteinases-2 (TIMP-2) through CCAAT sites (384). Results illustrated in Fig. 31 show that higher concentrations of E2 (> 10 nM) in the absence of cotransfected ER α induce luciferase activity this indicates that the endogenous ER α proteins are induced by E2 at higher concentration than when ER α is transfected. PKA expression plasmid and 8-Br-cAMP also induce reporter gene activity in ZR-75 cells transfected with pE2F-1 $_{j_{m1}}$. Moreover, hormonal or PKA-induced transactivation in ZR-75 cells transfected with this construct was not inhibited after cotransfection with 4YA13, whereas dominant negative NFYA (4YA13 $_{m29}$) significantly blocked both induction responses (Figure 32). These data are consistent with non-genomic activation of cAMP-PKA by E2 and

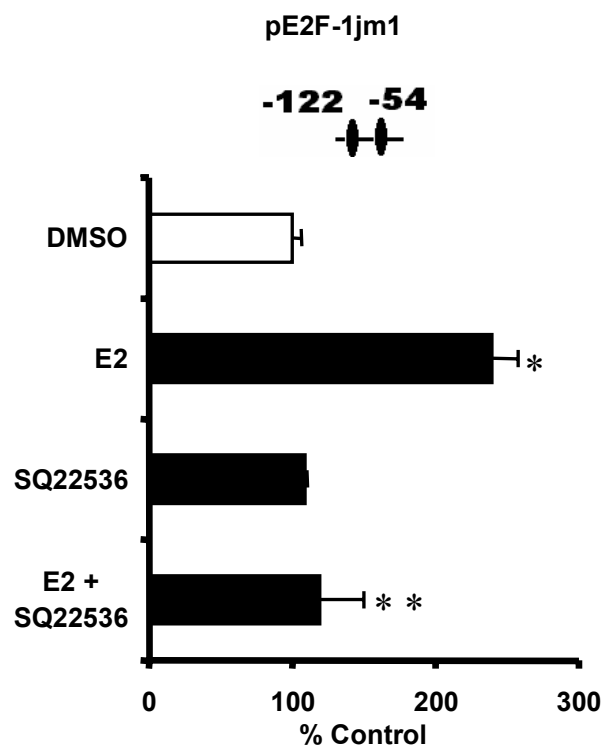


Fig. 30. Adenyl cyclase/cAMP -dependent inhibition of CCAAT sites in ZR-75 cells. Cells were transfected with pE2F-1_{jm1} and treated with E2 (+ cotransfected ER α) \pm 400 μ M SQ22536 and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction compared to DMSO is indicated by an asterisk and inhibition by a double asterisk. Results are means \pm SD of three replicate determinations for each treatment group. The DMSO treatments serve as a 100% control.

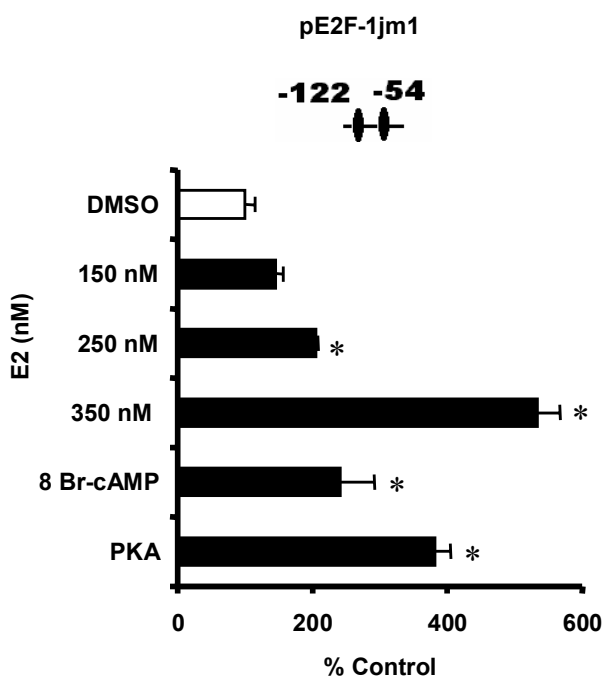


Fig. 31. cAMP/PKA-dependent activation of CCAAT sites or NFYA in ZR-75 cells. ZR-75 cells were transfected with pE2F-1_{jm1} and treated with 150-350 nM E2 alone (no cotransfected ER α), 800 μ M 8-bromocAMP or a PKA expression plasmid (1 μ g), and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction (*) or inhibition (**) is indicated. Results are means \pm SD of three replicate determinations for each treatment group. The DMSO treatments serve as a 100% control.

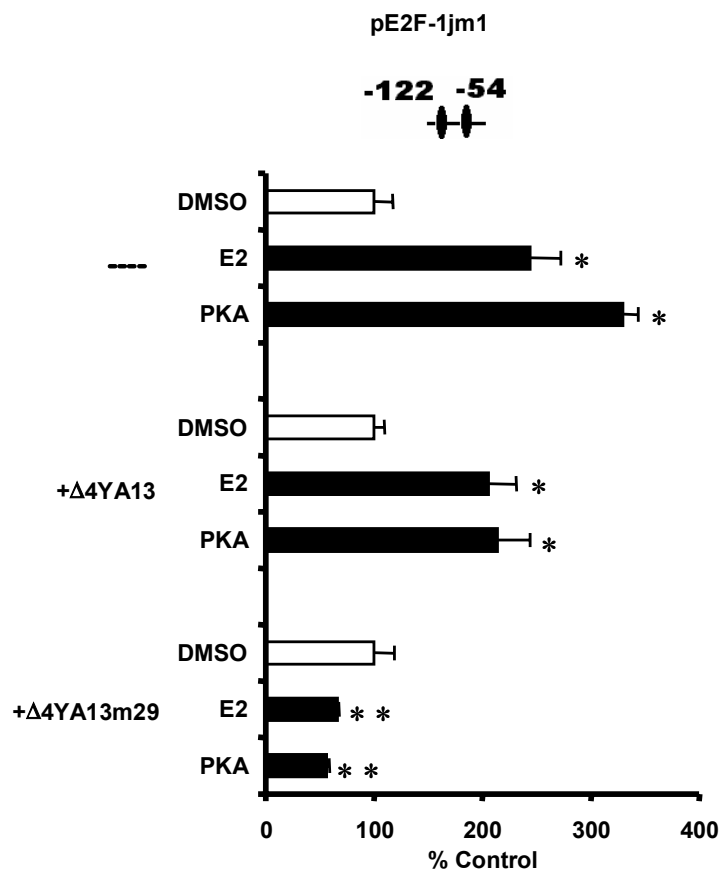


Fig. 32. Inhibition of PKA-dependent activation of pE2F-1_{jm1} by dominant negative NFYA ($\Delta 4YA13_{m29}$). ZR-75 cells were cotransfected with pE2F-1_{jm1}, ER α , the PKA expression plasmid (in the PKA group) and $\Delta 4YA13$ or $\Delta 4YA13_{m29}$ expression plasmid and treated with DMSO (DMSO and PKA), or 10 nM E2, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction (*) or inhibition (**) is indicated. Results are means \pm SD of three replicate determinations for each treatment group. The DMSO treatments serve as a 100% control.

subsequent downstream activation of NFYA. We also investigated hormonal activation of NFYA in ZR-75 cells transfected with an expression plasmid for a chimeric protein containing the DNA binding domain of the yeast GAL4 protein fused to NFYA (full length) (pM-NFYA) and a construct containing five tandem GAL 4 response elements linked to a bacterial luciferase reporter gene (pGAL4₅). E2 induced a 16-fold increase in reported gene activity which was inhibited in cells cotreated with E2 plus SQ22536 (Fig. 33). These results confirm that hormonal activation of the cAMP/PKA pathway in ZR-75 cells directly activates NFYA and this is consistent with the observed hormonal activation of constructs containing the CCAAT sites. Thus, hormonal activation of E2F-1 in ZR-75 cells involved both genomic ER α /Sp1 and non-genomic pathways and clearly differed from the genomic ER α /Sp1/NF-Y mechanism previously described in MCF-7 cells (342).

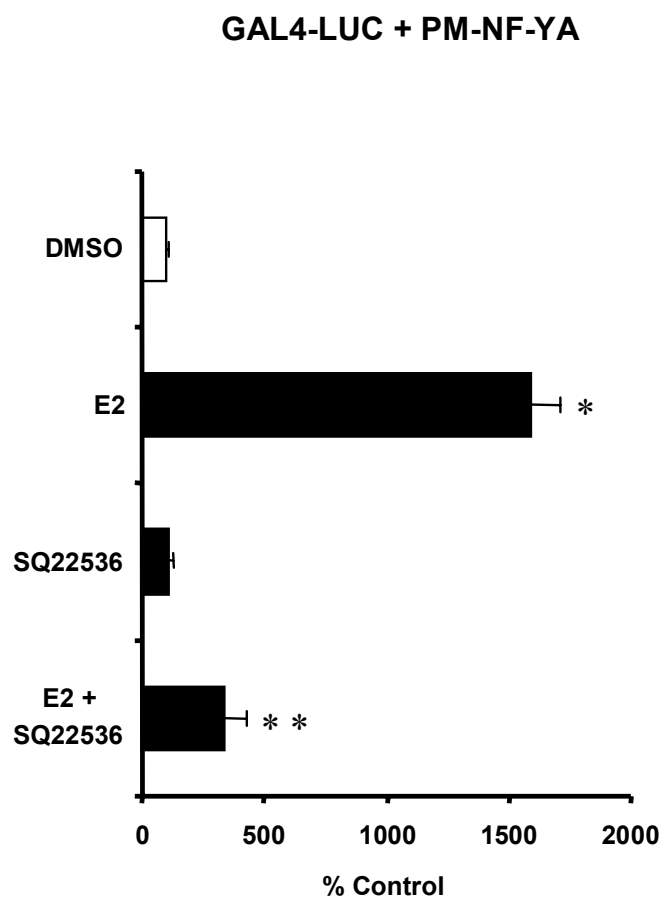


Fig. 33. Activation of pM-NFYA. ZR-75 cells were transfected with pM-NFYA/pGAL4₅, treated with DMSO, 10 nM E2, SQ22536 or E2 + SQ22536, and luciferase activity determined as described in the Materials and Methods. Significant ($p < 0.05$) induction (*) or inhibition (**) is indicated. Results are means \pm SD of three replicate determinations for each treatment group. The DMSO treatments serve as a 100% control.

3.2 Induction of E2F-1 Gene Expression in Breast Cancer Cells:

Mechanisms of Inhibition by Aryl Hydrocarbon Receptor Agonists

3.2.1 AhR/ER α Crosstalk in MCF-7 Cells Inhibits E2-induced E2F-1 Gene Expression

Previous studies in this laboratory have demonstrated that E2 induces E2F-1 protein expression in MCF-7 and ZR-75 cells (342, 343) and TCDD has been shown to inhibit E2-induced expression of multiple genes in breast cancer cells lines (262, 291, 292, 296, 331, 332, 365, 366). Inhibitory AhR-ER α crosstalk was investigated in both cell lines by treatment with DMSO, E2, TCDD, and E2 plus TCDD and the results for MCF-7 cells are illustrated in Figs. 34-36. Treatment of MCF-7 cells with E2 for 6, 12, or 24 h induced a time-dependent increase in E2F-1 protein as previously reported in this cell line whereas 10 nM TCDD either did not affect (6 and 12 h) or decreased E2F-1 protein expression (Fig. 34). In cells cotreated with E2 plus TCDD there was a significant decrease in hormone-induced E2F-1 protein levels after cotreatment for 12 and 24 h. Previous studies in MCF-7 cells show that E2 also induced E2F-1 mRNA levels and reporter gene activity in cells transfected with constructs containing E2F-1 gene promoter inserts that encompass the -169 to -54 promoter region (342). This minimal sequence binds ER α /Sp1 and NF-YA and forms a transcriptionally active ER α /Sp1-NF-YA complex. The results summarized in Figs. 35 and 36

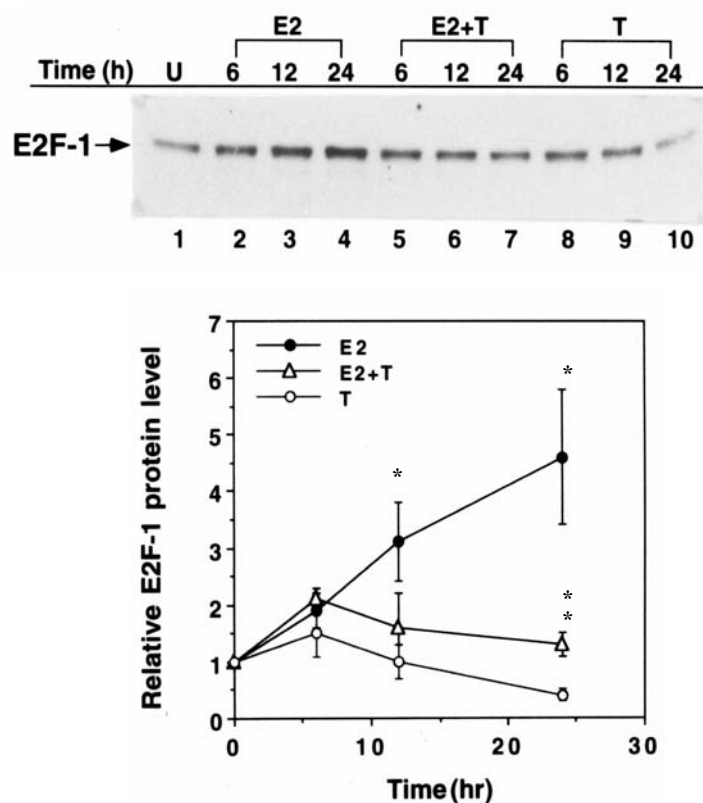


Fig. 34. TCDD downregulation of E2-induced protein expression in MCF-7 cells. MCF-7 cells were treated with 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 6, 12, and 24 h and E2F-1 protein levels were determined as described in Materials and Methods. E2F-1 protein levels in untreated cells (U) are also indicated. Results are normalized to Sp1 protein levels and expressed as means \pm SE for three replicate determinations for each treatment group. The relative protein value for the control group (U) set as 1 was the DMSO treatments.

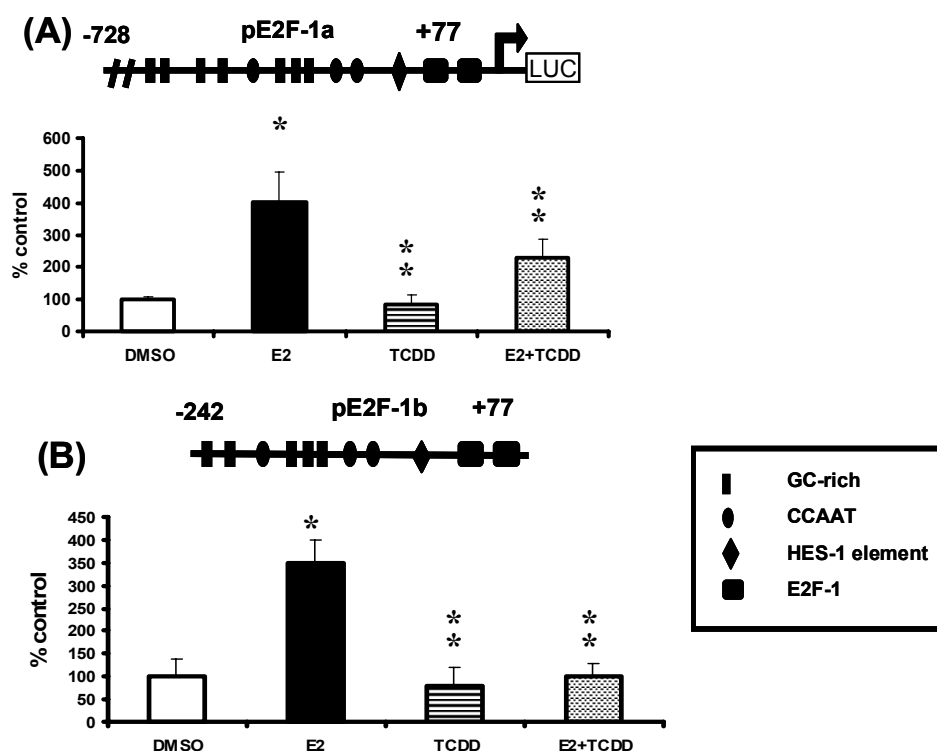


Fig. 35. Analysis of TCDD inhibition of E2 induced transactivation of E2F-1 promoter constructs in MCF-7 cells. Cells were transfected with constructs containing E2F-1 promoter inserts from the E2-responsive -728 to +77 region and treated with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 48 h and luciferase activity was determined as described in Materials and Methods. Significant induction by E2 is indicated with an asterisk (*), and inhibition is indicated by double asterisks (**). Results for (A)-(B) are expressed as means \pm SE for three replicate determinations for each treatment group. The 100% control groups were the DMSO treatments in cells transfected with pE2F-1a (A) and pE2F-1b (B).

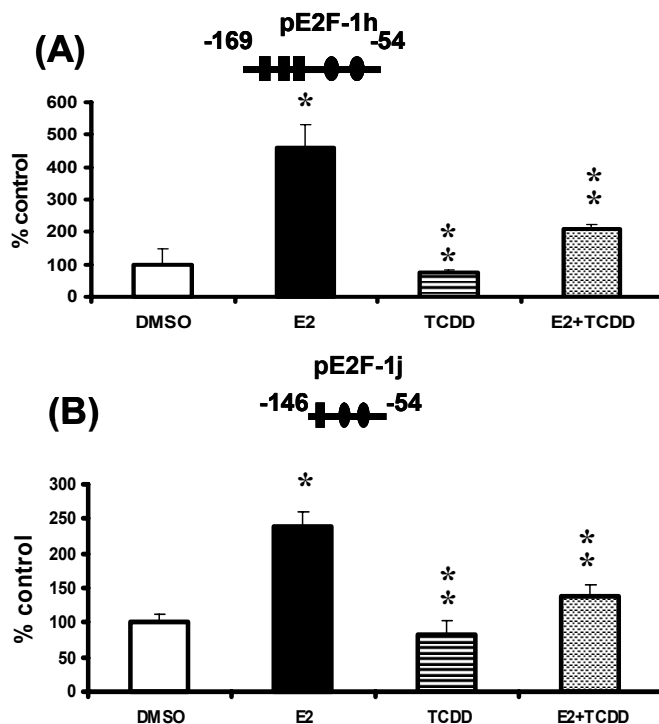


Fig. 36. Analysis of TCDD inhibition of E2 induced transactivation of E2F-1 minimal promoter region in MCF-7 cells. Cells were transfected with constructs containing E2F-1 promoter inserts from the E2-responsive -169 to -54 region and treated with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 48 h and luciferase activity was determined as described in Materials and Methods. Significant induction by E2 is indicated with an asterisk (*), and inhibition is indicated by double asterisks (**). Results for (A)-(B) are expressed as means \pm SE for three replicate determinations for each treatment group. The 100% control groups in (A)-(B) were the DMSO treatments in cells transfected with pE2F-1h (A) and pE2F-1j (B).

show that E2 induced transactivation in cells transfected with constructs containing -728 to +77 (pE2F-1a), -242 to +77 (pE2F-1b), -169 to 54 (pE2F-1h), and -146 to -54 (pE2F-1j) gene promoter inserts. TCDD (10 nM) alone did not induce transactivation in cells transfected with these constructs; however, in cells cotreated with TCDD plus E2 there was significant decrease in hormone-induced transactivation. These results are consistent with previous studies on inhibitory AhR-ER α crosstalk in MCF-7 cells where the combined treatment with TCDD and E2 results in limiting levels of ER α which is associated, in part with decreased nuclear ER α and ER α /Sp1-mediated transactivation (135, 368).

3.2.2 TCDD Inhibition of E2F-1 Gene Expression in ZR-75 Cells

Hormone-dependent regulation of E2F-1 gene expression in ZR-75 cells does not depend on formation of a nuclear ER α /Sp1-NF-YA complex since both the upstream GC-rich and downstream NF-YA binding sites alone are E2-responsive (343). Genomic ER α /Sp1 activates the upstream GC-rich sites whereas activation of downstream NF-YA is dependent on PKA-dependent non-genomic pathways of estrogen action. The results in Fig. 37 demonstrate that

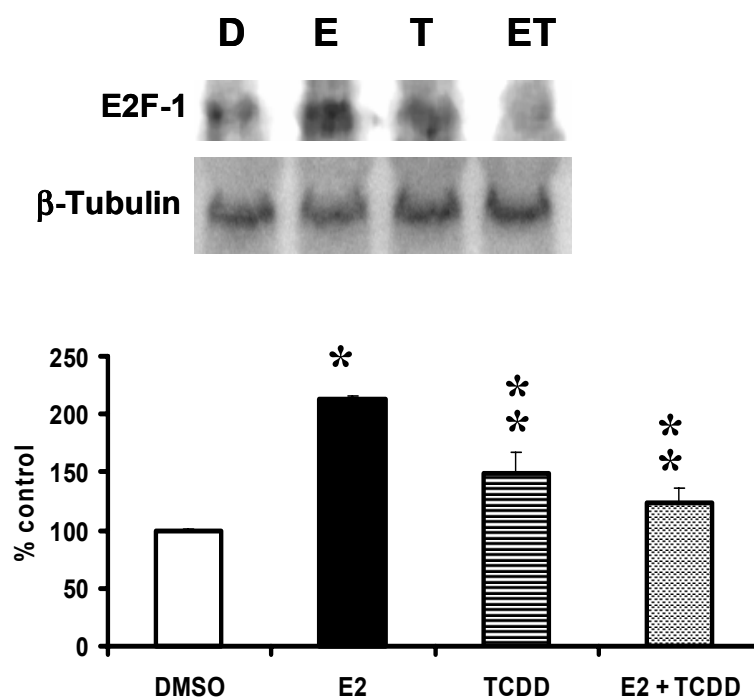


Fig. 37. Inhibition of E2-induced E2F-1 mRNA expression by TCDD in ZR-75 cells. Cells were treated with DMSO, 10 nM E2, 10 nM TCDD, and E2 plus TCDD for 12 h, and E2F-1 and β -tubulin mRNA levels were determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group. Induction by E2 is indicated by an asterisk (*) and inhibition by TCDD is indicated by double asterisks (**).

E2 induces E2F-1 mRNA levels and TCDD alone also slightly increased E2F-1 mRNA expression. However, in ZR-75 cells treated with TCDD plus E2 there was significant decrease in the hormone-induced response. Using the same treatment protocol it was evident that TCDD also inhibited E2-induced E2F-1 protein expression in ZR-75 cells within 4 hours after treatment (Fig. 38) and similar results were observed at longer time points (data not shown).

A series of constructs containing GC-rich sites alone or in combination with the downstream NFY site were used in transient transfection studies to investigate inhibitory AhR-ER α crosstalk associated with genomic ER α /Sp1 action in ZR-75 cells (Figs. 39 and 40). The results showed that E2 induced activity in ZR-75 cells transfected with pE2F-1a, pE2F-1j, pE2F-1j_{m2} and pE2F-1k (-169 to -111) and cotreatment with TCDD plus E2 significantly decreased hormone-induced activity. These results are consistent with the data in MCF-7 cells given in Figures 34-36 showing that inhibitory AhR-ER α crosstalk that involves genomic ER α /Sp1 is associated with decreased E2F-1 expression in the cotreatment (TCDD plus E2) group.

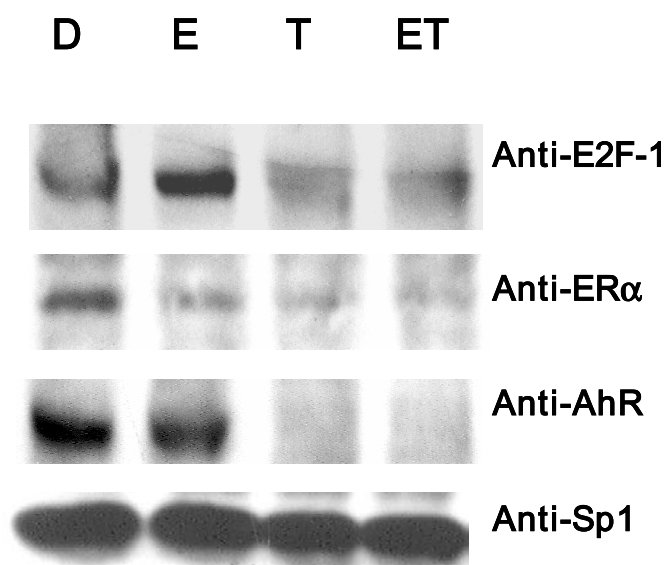


Fig. 38. Inhibition of E2-induced E2F-1 protein expression by TCDD in ZR-75 cells. Cells were treated with DMSO, 10 nM E2, 10 nM TCDD, and TCDD plus E2 for 4 h. E2F-1, ER α , AhR, and Sp1 levels were determined as described in Material and Methods. Sp1 was used as a loading control for these experiments.

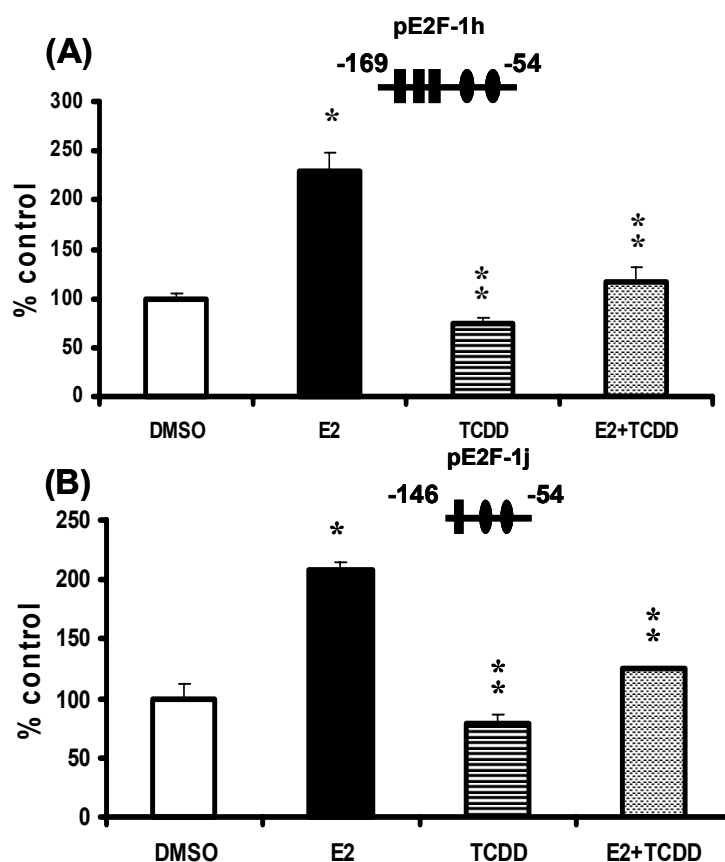


Fig. 39. Inhibitory AhR/ER crosstalk on E2F-1 constructs in ZR-75 cells. ZR-75 cells were transiently transfected with E2F-1 promoter constructs pE2F-1h (A) and pE2F-1j (B) and treated with DMSO, E2, TCDD and their combination for 48 h and luciferase activity was determined as described in Materials and Methods. Significant ($p < 0.05$) induction is indicated with an asterisk (*) and inhibition is indicated by double asterisks (**), and results are expressed as means \pm SE for three replicate determinations for each treatment group.

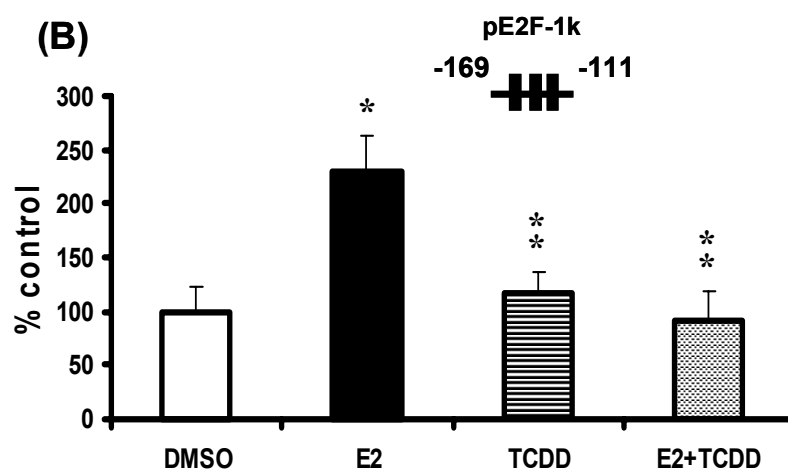


Fig. 40. Inhibitory AhR-ER α crosstalk on the E2F-1 involves genomic ER α /Sp1. ZR-75 cells were transiently transfected with E2F-1 promoter constructs pE2F-1j_{m2} (A) and pE2F-1k (B) and treated with DMSO, E2, TCDD and their combination for 48 h and luciferase activity was determined as described in Materials and Methods. Significant ($p < 0.05$) induction is indicated with an asterisk (*), inhibition is indicated by double asterisks (**), and results are expressed as means \pm SE for three replicate determinations for each treatment group.

3.2.3 AhR-mediated Inhibition of E2-induced Protein Expression of E2F-1 Does Not Involve Proteasome Degradation

Previous studies in this laboratory showed that combined treatment of MCF-7 or ZR-75 cells with E2 plus TCDD resulted in limiting levels of ER α expression which correlated with inhibition of E2-induced transactivation (using an ERE promoter) and Fos protein expression by TCDD (135, 368). Moreover, inhibition of proteasome-dependent degradation of ER α protein by MG132 partially reversed the inhibitory effects of TCDD. We therefore investigated the role of limiting levels of ER α on inhibitory AhR-ER α crosstalk associated with E2F-1 in MCF-7 (Fig. 41) and ZR-75 (Fig. 42) cells. E2 induced E2F-1 protein and TCDD inhibited this response in both cell lines and this was accompanied by decreased ER α (E2 and E2 plus TCDD treatment groups) and AhR (TCDD and E2 plus TCDD treatment groups) proteins as previously reported (135, 368). Pretreatment of the cells with the proteasome inhibitor MG132 blocked ligand-induced downregulation of ER α and AhR. However, inhibition of proteasomes also prevented hormone-induced upregulation of E2F-1 protein and levels were unchanged in all treatment groups. Because of the unexpected effects of MG132 on hormone-induced E2F-1, this does not exclude a role TCDD limiting the levels of ER α available for E2-induced transactivation in inhibitory AhR-ER α crosstalk.

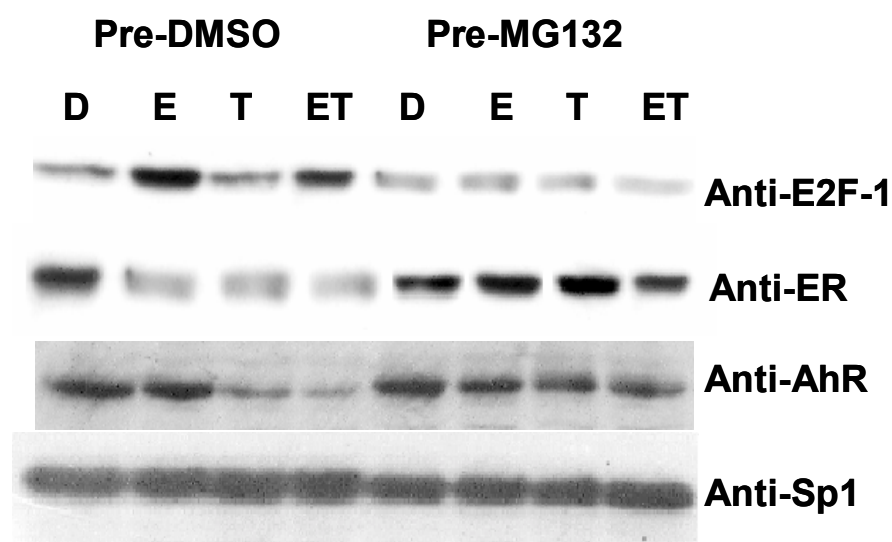


Fig. 41. The role of proteasome degradation in AhR-mediated inhibition of E2-induced E2F-1 protein expression in MCF-7 cells. MCF-7 cells were pretreated with 10 μ M MG132 and DMSO for 30 min and then treated with DMSO, E2, TCDD, and E2 plus TCDD for 6 h. E2F-1, ER α , AhR, and Sp1 levels were determined as described in Material and Methods. Sp1 was used as a loading control for these experiments.

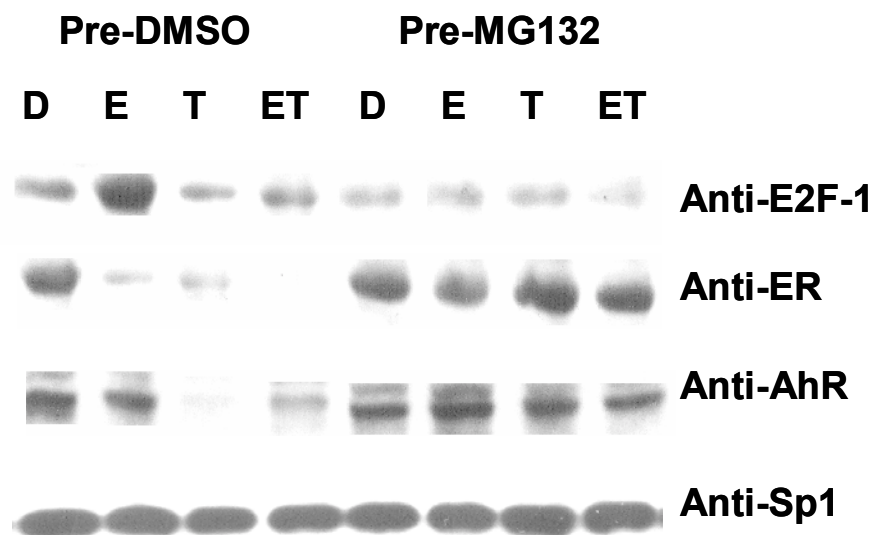


Fig. 42. Inhibition of proteasome degradation in AhR-mediated inhibition of E2-induced E2F-1 protein expression in ZR-75 cells. ZR-75 cells were pretreated with 10 μ M MG132 and DMSO for 30 min and then treated with DMSO, E2, TCDD, and E2 plus TCDD for 6 h. E2F-1, ER α , AhR, and Sp1 levels were determined as described in Material and Methods. Sp1 was used as a loading control for these experiments.

3.2.4 Inhibitory AhR/ER α Crosstalk in Schneider Cells

SL-2 Schneider cells do not express Sp1, ER α , or the AhR, and functional interactions of these transcription factors were further investigated in this cell line transfected with pE2F-1h (Figs. 43-45). Basal activity in cells transactivated with pE2F-1h alone was set at 100% and cotransfection with 250 ng Sp1 expression plasmid caused a maximal (>350-fold) increase in activity (Fig. 43A). In contrast, transfection with ER α caused a minimal (<3-fold) increase in luciferase activity (Fig. 43B). Cotransfection of the AhR/Arnt complex caused only minimal activation of pE2F-1h (<5-fold) and in combination with Sp1 the effects were less than additive and TCDD did not significantly enhance transactivation (Figs. 43C and 44B). This activity was not E2-responsive since ER α does not directly interact with the E2F-1 gene promoter (342, 343). In cells treated with E2 or DMSO and cotransfected with both Sp1 and ER α expression plasmids, cotransfection with Sp1 increased basal activity and E2 slightly induced activity in cells transfected with 25 or 50 ng ER α expression plasmid and significant induction (ca 2-fold) was observed using higher amounts (100-1000 ng) of ER α expression plasmid (Fig. 44A). The results demonstrate that in SL-2 cells ER α /Sp1 is a functional ligand-activated transcription factor that in turn activates GC-rich promoters. TCDD-dependent inhibitory AhR-ER α crosstalk was also investigated in SL-2 cells transfected with

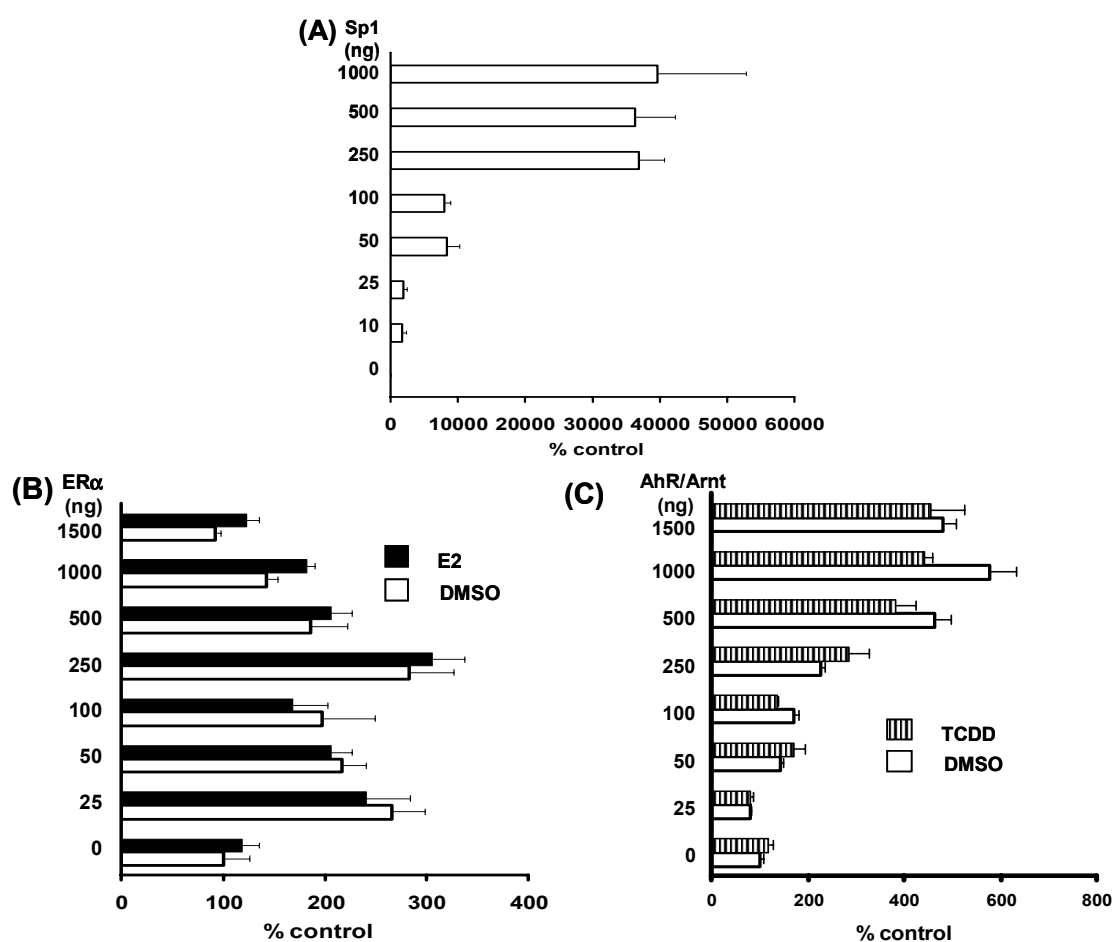


Fig. 43. Activation of pE2F-1h by ER α , Sp1, and AhR/Arnt. Transfection of pE2F-1h into SL-2 cells. Cells were transfected with pE2F-1h and pPac-Sp1 (A), pPAC-ER α (B) and pPAC-AhR and pPAC-Arnt (C). The cells were then treated with DMSO, 10 nM E2, or 10 nM TCDD for 48 h. Luciferase activity was determined as described in Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk (*) and inhibition by double asterisks (**) and results are expressed as means \pm SE for three replicate determinations for each treatment group.

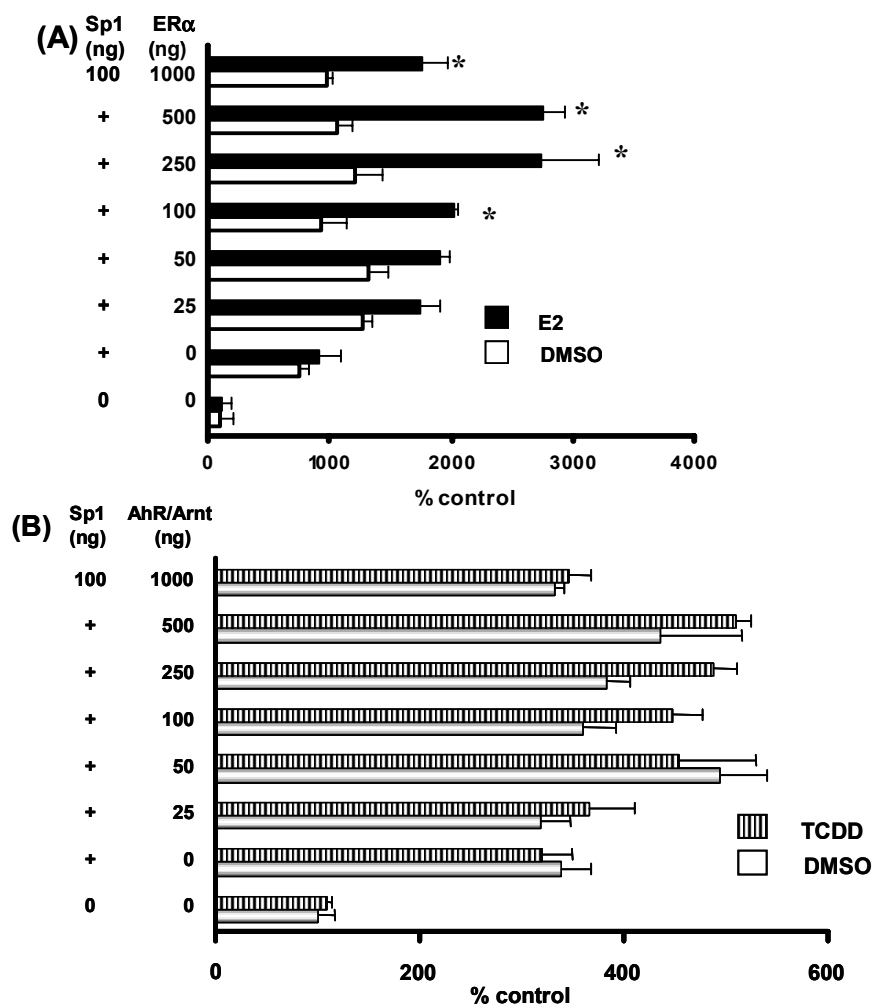


Fig. 44. Sp1 interactions with ER α and AhR. SL-2 cells were transfected with pE2F-1h and titrated with 100 ng pPac-Sp1 plus pPAC-ER α (A) and 100 ng pPac-Sp1 plus pPAC-AhR and pPAC-Arnt (B). The cells were then treated with DMSO, 10 nM E2, or 10 nM TCDD for 48 h and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk (*) and results are expressed as means + SE for three replicate determinations for each treatment group.

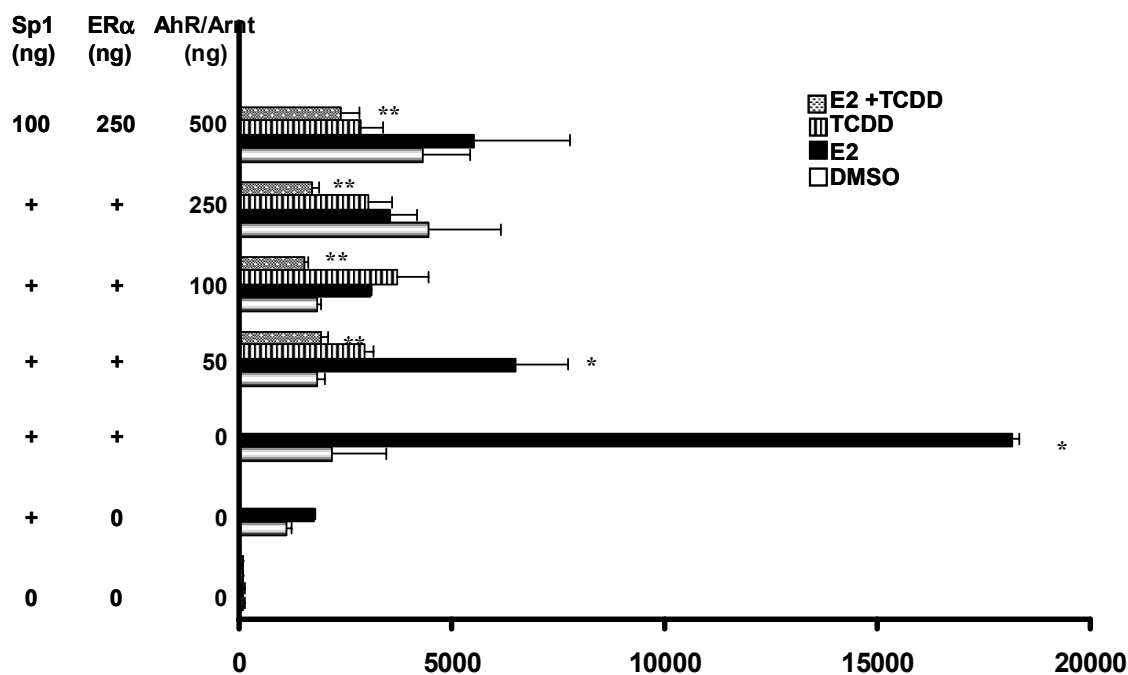


Fig. 45. TCDD-dependent inhibition of hormone-induced pE2F-1h in SL-2 cells. SL-2 cells were transfected with pE2F-1h and titrated with 100 ng pPAC-Sp1, 250 ng pPAC-ER α , plus different amounts of pPAC-AhR and pPAC-Arnt. The cells were then treated with DMSO, 10 nM E2, 10 nM TCDD and their combination for 48 h and luciferase activity was determined as described in Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk (*), inhibition is indicated by double asterisks (**) and the results are expressed as means + SE for three replicate determinations for each treatment group.

ER α , Sp1, and AhR/Arnt expression plasmids, pE2F-1h and treated with DMSO, TCDD, E2 or their combination (Fig. 45). E2-responsiveness was observed in cells treated with E2 and transfected with ER α and Sp1 and after cotransfection with 50 ng AhR/Arnt the E2-induced response for the E2 group was decreased and cotreatment with TCDD further inhibited this response. Thus inhibitory AhR-ER α crosstalk could also be observed in SL-2 cells transfected with pE2F-1h and this was comparable to results obtained for the same constructs in ZR-75 cells (Figs. 39 and 40). In contrast, cotransfection with increasing amounts of AhR/Arnt did not significantly affect transactivation in cells treated TCDD alone or TCDD plus E2. However, E2-induced transactivation was slightly decreased and this was accompanied by increased luciferase activity in DMSO (control) treated cells. Even though E2-responsiveness is lost after transfection with higher amounts of AhR/Arnt (100-500 ng), luciferase activity was significantly decreased in the TCDD plus E2 group compared to the E2-treated group in SL-2 cells transfected with 50, 100, 250, or 500 μ g AhR/Arnt. These results are consistent with the activity of AhR/Arnt acting as a ligand-dependent repressor of ER α /Sp1.

3.2.5 TCDD Inhibition of E2F-1 Gene Expression through the cAMP/PKA Pathway

E2-dependent induction of E2F-1 in ZR-75 cells results in activation of NF-YA through non-genomic pathways associated with activation cAMP/PKA. A recent study showed that TCDD induced the transcriptional repressor HES-1 in T47D breast cancer cells and HES-1 inhibits E2-induced E2F-1 through direct interactions with a HES-1 response element in the E2F-1 promoter (388, 389). In order to determine if HES-1 played a role in inhibitory AhR-ER α cross talk, ZR-75 cells were transfected with pE2F-1d which contains two CCAAT sites, the HES-1 motif and two downstream E2F sites within the -122 to +77 region of the E2F-1 promoter. E2 induced luciferase activity of pE2F-1d (Fig. 46A). Only minimal induction of HES-1 by TCDD was observed in ZR-75 cells (data not shown) and in cells transfected with pE2F-1j, TCDD significantly inhibited hormone-induced transactivation (Fig. 46B). The -122 to -54 E2F-1 promoter sequence which is contained in pE2F-1j does not contain the HES-1 response element (-41 to -36) indicating that inhibitory AhR-ER α crosstalk is HES-1-independent and associated with interaction with the cAMP/PKA signaling pathway. Western blot analysis of whole cell lysates from ZR-75 cells treated with DMSO, E2, TCDD or TCDD plus E2 for 2 h in order to investigate the role of TCDD in E2-induced PKA phosphorylation. TCDD inhibited hormone-induced E2F-1 protein expression and PKA phosphorylation whereas PKA and Sp1

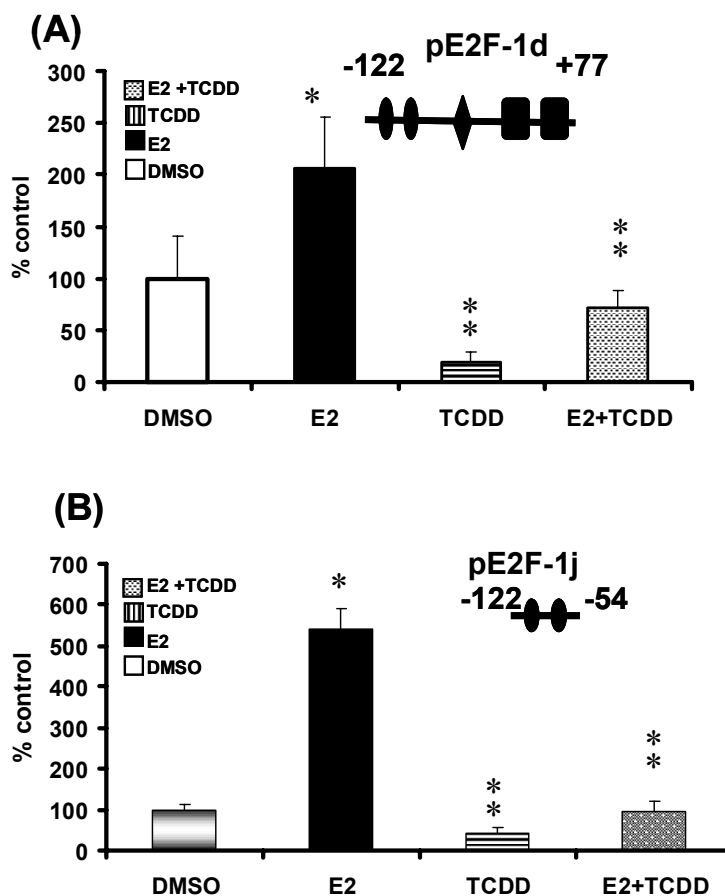


Fig. 46. AhR-dependent inhibition of E2F-1 transactivation is HES-1 independent in ZR-75 cells. Cells were transfected with pE2F-1d and pE2F-1j and treated with DMSO, 10 nM E2, 10 nM TCDD and their combination for 48 h and luciferase activity was determined as described in Materials and Methods. The results are expressed as means \pm SE of three replicate determinants for each treatment group and significant ($p < 0.05$) induction is indicated by an asterisk (*) and inhibition by double asterisks (**).

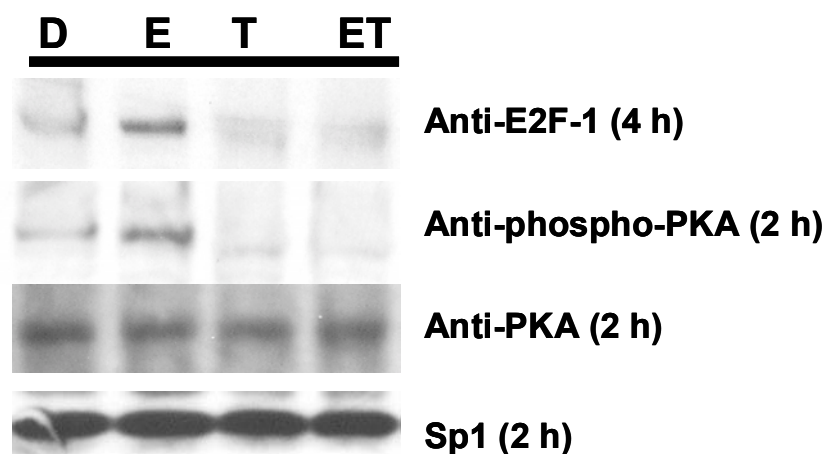


Fig. 47. Inhibition of hormone-induced E2F-1 protein and PKA phosphorylation by TCDD. Cells were treated for 2 to 4 h with DMSO, E2, TCDD and TCDD plus E2 and E2F-1, phospho-PKA, PKA, and Sp1 protein levels were determined as described in Materials and Methods.

(loading control) proteins were unchanged in all treatment groups (Fig. 47). PKA activity was also inhibited by TCDD in cells treated with 8-bromo-cAMP or transfected with a constitutively- active PKA expression plasmid (Fig. 48). Previous studies have demonstrated that like TCDD, the adenylyl cyclase inhibitor SQ22356 also blocked hormone-induced transactivation in ZR-75 cells transfected with pE2F-1j and we therefore compared the activity of TCDD and SQ22356 as inhibitors of cAMP production in ZR-75 cells (Table 4). cAMP levels varied from 1.5 - 17 pmol/mg and after treatment with E2 for 30, 60, or 120 min cAMP levels significantly increased to 6.0 ± 0.1 , 4.8 ± 0.2 and 3.6 ± 0.5 pmol/mg respectively. TCDD alone had no effect on the cAMP levels. However, in cells treated with TCDD plus E2, hormone-induced cAMP was completely suppressed at the three time points. Moreover, similar inhibitory effects were observed for SQ22356 suggesting that AhR-mediated inhibition of the hormone-induced cAMP/PKA pathway in ZR-75 cells is linked to direct inhibition of adenylyl cyclase activity and this inhibition subsequently affects E2F-1 transactivation through NF-YA (Fig. 49). This represents the first example of AhR-dependent inhibition of non-genomic pathways of ER action and demonstrates that inhibitory AhR-ER α crosstalk associated with E2F-1 involves inhibition of both genomic and non-genomic pathways of estrogen action.

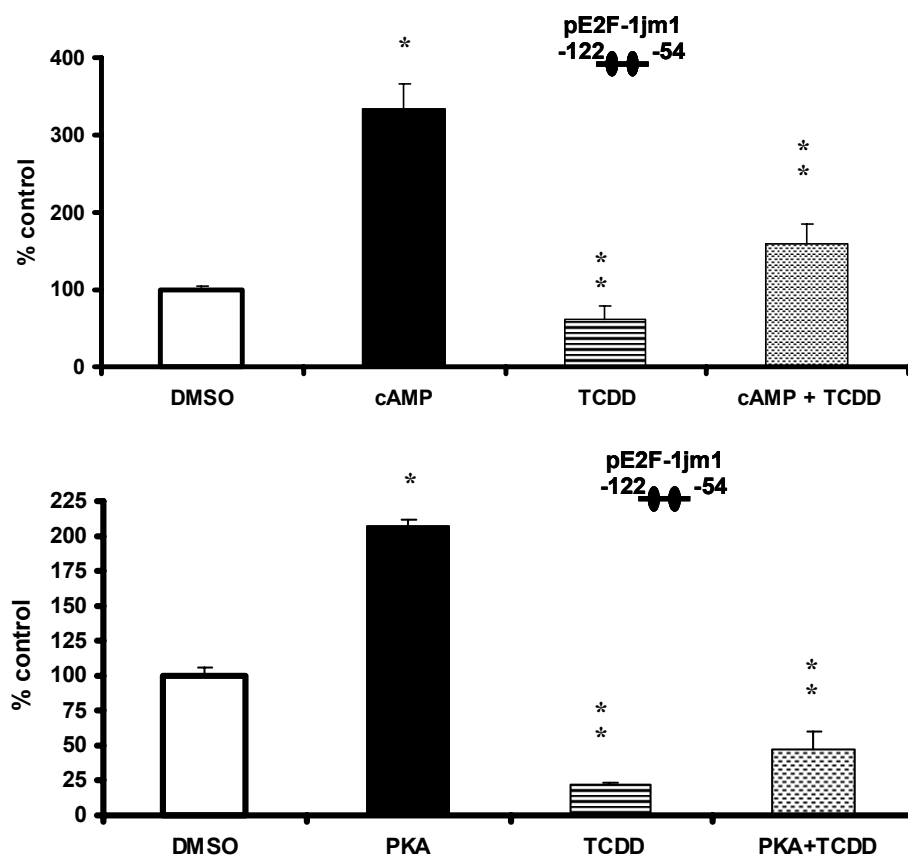


Fig. 48. Inhibitory AhR/ER α effects on cAMP/PKA transactivation of the pE2F-1j_{m1}. (A). Inhibition of 8 Br-cAMP induced E2F-1 transactivation by TCDD. Cells were transfected with pE2F-1g and treated with DMSO, 1 μ M 8 Br-cAMP, TCDD, and TCDD plus 8 Br-cAMP for 48 hrs. (B) ZR-75 cells were co-transfected with a PKA expression plasmid and treated with DMSO and TCDD. Luciferase activity was determined as described in Materials and Methods. The results are expressed as means \pm SE of three replicate determinants for each treatment group and significant ($p < 0.05$) induction is indicated by an asterisk (*) and inhibition by double asterisks (**).

Table 4. *cAMP* enzyme immunoassay (EIA) ZR-75 cells^a.

| Treatment | cAMP levels (pmol/mg protein) | | |
|--------------|-------------------------------|-------------|-------------|
| | 30 min | 60 min | 120 min |
| control | 1.7 ± 0.1 | 1.6 ± 0.1 | 1.5 ± 0.1 |
| E2 | 6.6 ± 0.1* | 4.8 ± 0.2 | 3.6 ± 0.5* |
| TCDD | 1.7 ± 0.4** | 1.8 ± 0.3** | 1.8 ± 0.2** |
| E2 + TCDD | 1.8 ± 0.2** | 1.6 ± 0.4** | 2.0 ± 0.6** |
| SQ22536 | 1.8 ± 0.1** | 1.8 ± 0.5* | 1.9 ± 0.1** |
| E2 + SQ22536 | 2.0 ± 0.7** | 1.9 ± 0.1** | 1.5 ± 0.5** |

^aZR-75 cells were treated for different times as cAMP levels were determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by E2 and inhibition by TCDD or SQ22536 (**) are indicated.

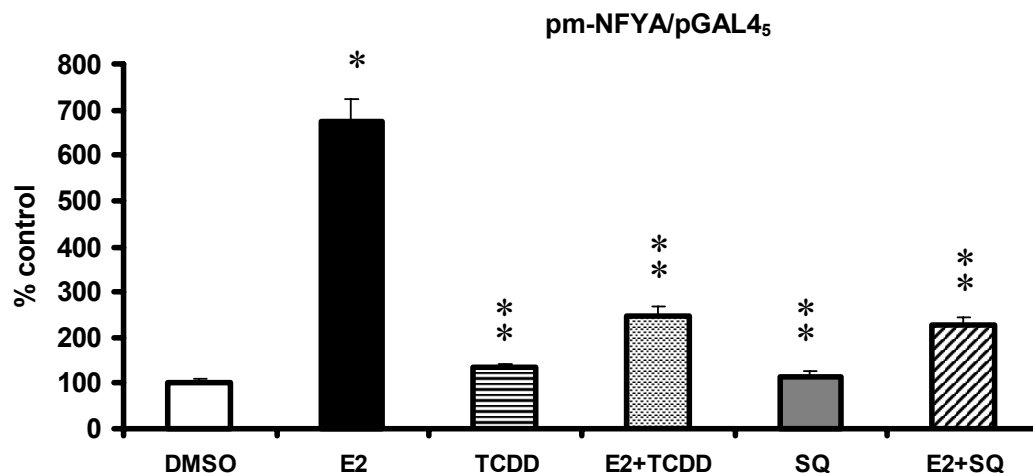


Fig. 49. Inhibition of hormone-induced activation of pM-NFYA by TCDD. ZR-75 cells were transfected with pM-NFYA/pGal4₅ and treated with DMSO, 10 nM E2, 10 nM TCDD, TCDD plus E2, 400 μ M SQ22536, and E2 plus SQ22536 and luciferase activity was determined as described in Materials and Methods. Results are means \pm SE of three replicate determinants for each treatment group and significant ($p < 0.05$) induction is indicated by an asterisk (*) and inhibition by double asterisks (**).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

4.1 Cell Context-dependent Differences in the Hormonal Activation of E2F-

1 Gene Expression

E2F transcription factors play a critical role in cell growth and are key regulators of $G_1 \rightarrow S$ phase progression of the cell cycle (349-352). E2F plays a paradoxical role in carcinogenesis and exhibits characteristics consistent with oncogenic and tumor suppressor activity (348-351). E2F-1^{-/-} and E2F-1^{+/-} mice are viable and, surprisingly, exhibit age-dependent hyperplastic and neoplastic responses suggesting some tumor suppressor activity (390, 391). E2F-1 also exhibits oncogenic activity and E2F-1 enhances formation of some tumors in mice lacking a functional Rb1 allele (392, 393). Using a transgenic murine model in which overexpression of E2F-1 is controlled by the keratin 5 promoter, it was reported that E2F-1 alone or in combination with other factors enhanced development of skin carcinomas and tumors in many other tissues (394-396). In contrast, these transgenic mice were resistant to development of skin tumors in a two-stage initiation-promotion model (396). Thus, E2F-1 overexpression can result in both oncogenic and tumor suppressive activity in the same tissue.

MCF-7 and ZR-75 are ER-positive breast cancer cell lines which are extensively used as models for investigating cell context-dependent differences in the molecular mechanisms of hormone-induced transactivation. For example, in ZR-75 cells E2 induces transactivation in cells transfected with constructs containing GC-rich proximal promoter inserts from the VEGF gene, whereas E2 decreases activity in MCF-7 and HEC1A cells (260) transfected with the same constructs. Previous studies have reported that E2 induces E2F-1 mRNA levels in MCF-7 cells (342), and similar results were observed in ZR-75 cells (Fig. 19). Deletion and mutation analysis of a series of constructs containing the -169/-173 to -54 region of the E2F-1 promoter clearly demonstrate cell context-dependent differences in hormonal activation of these constructs in MCF-7 vs. ZR-75 cells (Figs. 20-22). In the former cell line, one or more of the GC-rich motifs (-169 to -111) and both CCAAT elements (-122 to -54) are required for ER α /Sp1/NFYA interactions on this promoter (Fig. 50). This same complex may play some role in hormone-induced transactivation in ZR-75 cells; however, the results clearly demonstrate that the GC-rich sites alone (pE2F-1k) or the CCAAT sites alone (pE2F-1j_{m1}) are sufficient for hormone activation. Gel mobility shift assays exhibit similar patterns of Sp1 and NFYA binding to the proximal region of the E2F-1 gene promoter in MCF-7 and ZR-75 cells (Figs. 23-27); however, there were clear differences in transactivation by ER α mutants in the two cell lines (Figs. 23 and 24). In ZR-75 cells, hormone-induced transactivation was

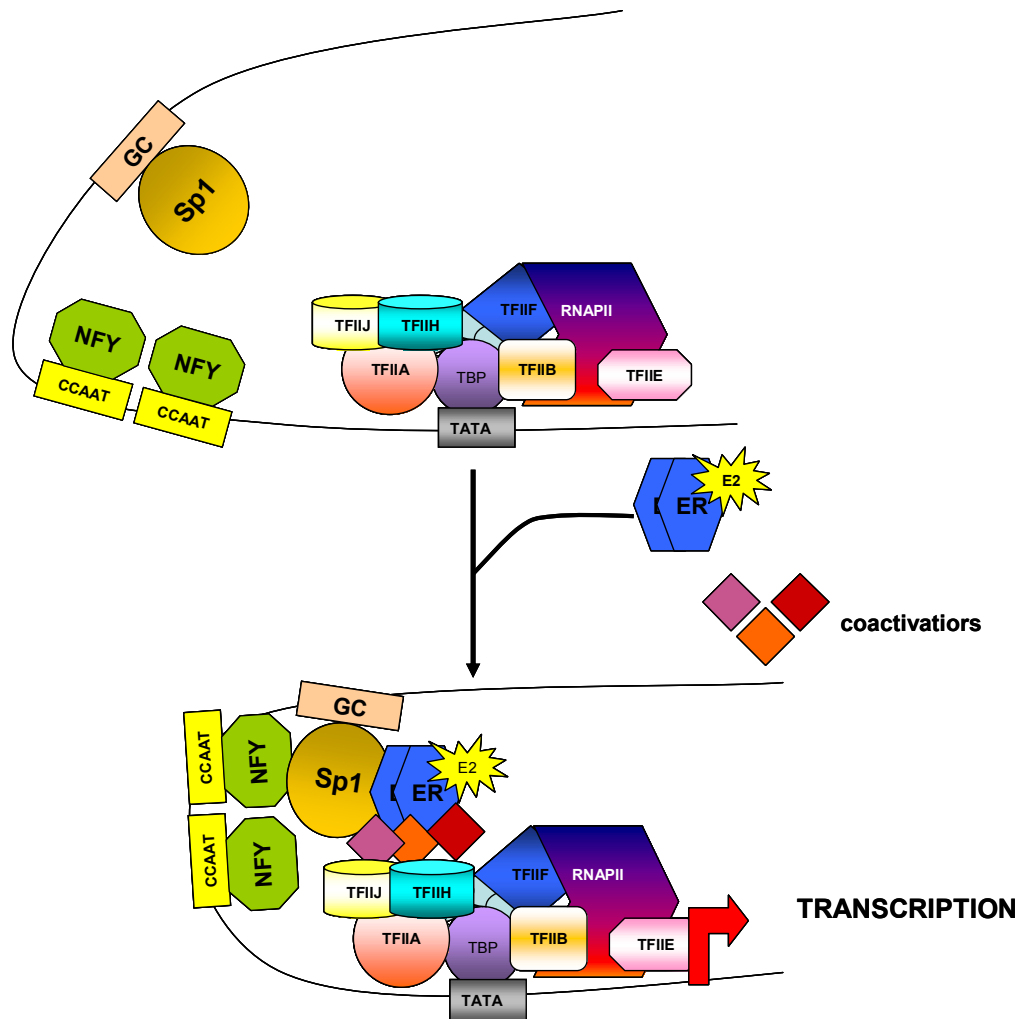


Fig. 50. The ER α /Sp1/NFY complex in MCF-7 cells. The NFY proteins bind to the CCAAT motifs and Sp1 binds to the GC-rich motifs. Upon E2 treatment, ER α heterodimerizes and forms an ER α /Sp1/NFY complex and interacts with coactivators that allow interactions with the basal transcription machinery to activate transcription of the E2F-1 gene in MCF-7 cells.

observed in cells transfected with the DBD-mutant HE11 and constructs containing GC-rich sites and mutated CCAAT sites (-146/-54) alone or in combination with CCAAT motifs (Fig. 24). These results are consistent with activation of GC-rich constructs by ER α /Sp1 observed for other E2-responsive gene promoters in ER-positive breast cancer cells (141, 172-176, 370, 371). In contrast, transfection with HE11 does not activate pE2F-1h or pE2F-1j (Fig. 23) in MCF-7 cells, and this differentiates between the transcriptionally-active ER α /Sp1/NFYA complex formed in MCF-7 cells (Fig. 50) in which the GC-rich site alone is not hormone-responsive (Fig. 22), whereas in ZR-75 cells, both GC-rich and CCAAT sites alone are hormone-responsive (Figs. 21 and 22).

ER α /Sp1-mediated activation of GC-rich motifs has been characterized in several E2-responsive gene promoters (141, 172-176, 370, 371), and hormone-dependent activation of constructs containing only the GC-rich sequences (i.e. pE2F-1j_{m2} and pE2F-1k) in ZR-75 cells is not surprising. The failure to activate the GC-rich constructs from the E2F-1 gene promoter in MCF-7 cells (Figs. 20-22) suggests that the cell context-dependent differences between ZR-75 and MCF-7 cells must be related in part, to specific regions within this GC-rich promoter. Although the E2F-1-derived GC-rich constructs alone were not hormone-responsive in MCF-7 cells, there are many other examples of GC-rich promoter constructs that are induced by E2. For example, the *bcl-2* gene has two regions that are important for transactivation (175). The upstream region

contains GC-rich sequences that were shown to interact with Sp1 in gel shift mobility assays and *in vitro* footprinting. Transcriptional activation of *bcl-2* promoter constructs containing GC-rich sites was induced by E2 through ER α /Sp1 interactions (175). Several other genes that are induced by E2 in MCF-7 cells are activated through one or more GC-rich sites in their regulatory regions and these include the cathepsin D (365, 367), c-fos (173), adenosine deaminase (174, 210), cad (178), and retinoic acid receptor alpha 1 (370) genes (Fig. 51). However, there are several other genes that contain one or more proximal GC-rich promoter elements that also bind Sp1 but are not hormone-responsive in transactivation assays, and some of these include ornithine decarboxylase (218), lactate dehydrogenase-A (219), and *p53* (217) genes (Fig. 51). Studies in this laboratory have also demonstrated ligand-dependent differences in activation of GC-rich constructs in MCF-7 and ZR-75 cells (141, 234, 262). For example, E2 and antiestrogens activate a construct (pSp1₃) containing three tandem consensus GC-rich sites whereas GC-rich promoters from several E2-responsive genes are activated by E2 but not antiestrogens. ICI 182,780 and 4-OH-tamoxifen both induced transactivation of pSp1₃ in MCF-7 and ZR-75 cells yet they both inhibited E2-induced transactivation of E2F-1 promoter constructs containing the upstream GC-rich motifs with or without the downstream CCAAT motifs (Figs. 52 and 53 unpublished data, Ngwenya 2004). Current studies are focused on identifying motifs within the GC-rich region of the

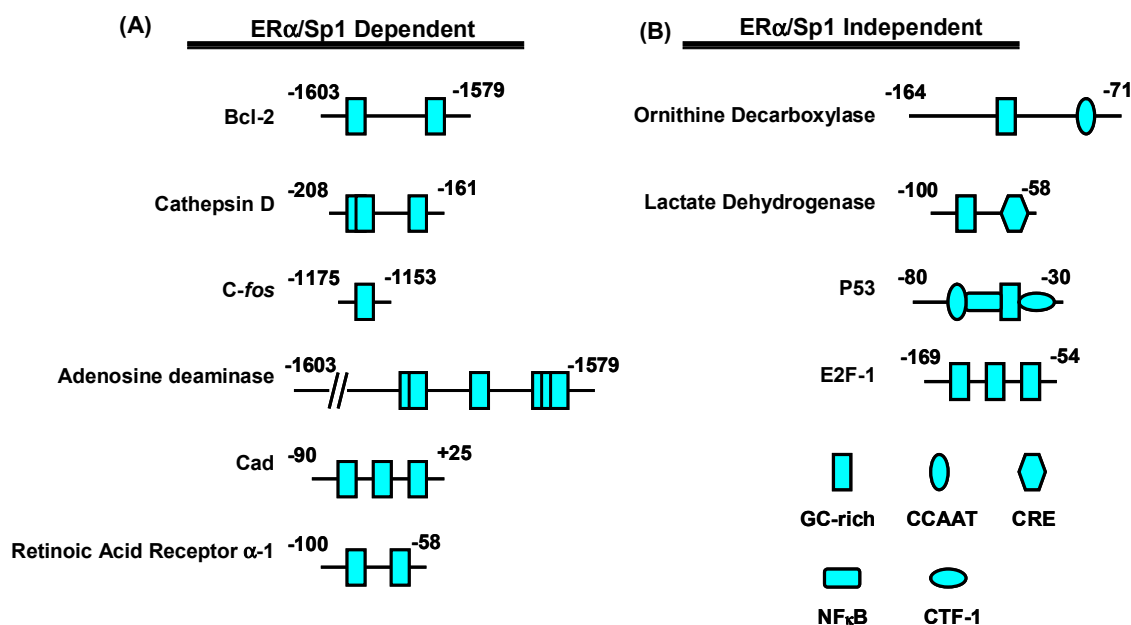


Fig. 51. GC-rich gene promoter constructs that are ERα/Sp1-dependent or – independent in MCF-7 cells. (A) Promoter constructs of genes that have at least one GC-rich site and undergo ERα/Sp1-mediated transactivation in MCF-7 cells. (B) Promoter constructs of genes that have at least one GC-rich site but are not transactivated in an ERα/Sp1-dependent manner.

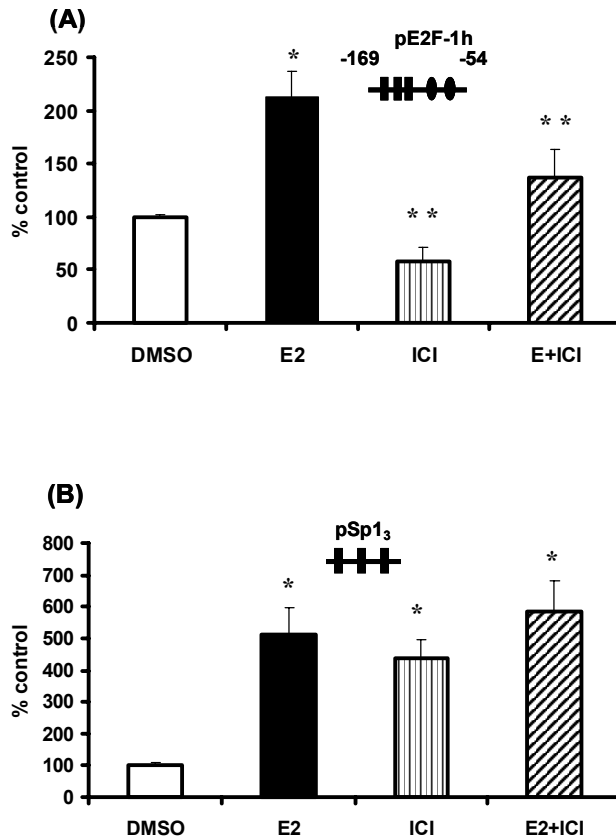


Fig. 52. Comparative analysis of ICI 182,780 treatment on transactivation in MCF-7 cells. MCF-7 cells were transfected with pE2f-1h or pSp1₃ and treated with DMSO, E2 , 10 nM ICI, and E2 plus ICI. Luciferase activity was determined as described in Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk (*), inhibition is indicated by double asterisks (**) and the results are expressed as means + SE for three replicate determinations for each treatment group.

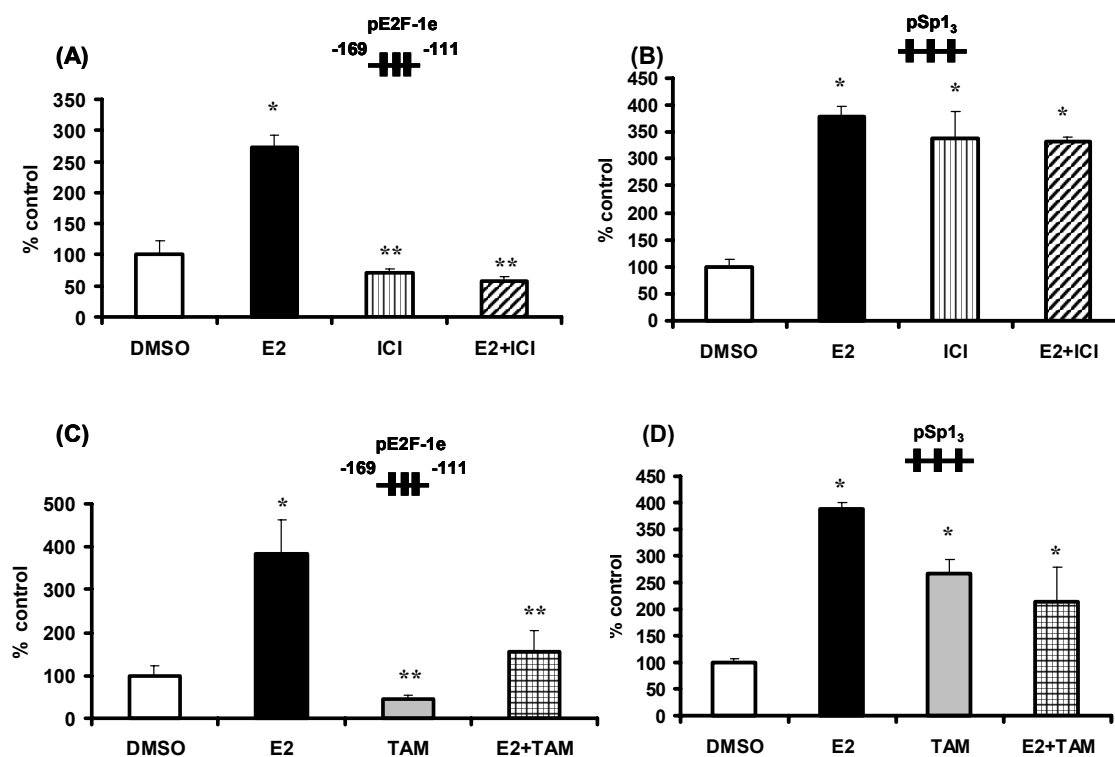


Fig. 53. The effects of ICI 182,780 and 4-OH-tamoxifen on transactivation of GC-rich promoters in ZR-75 cells. ZR-75 cells were transfected with pE2F-1k or pSp1₃ and treated with DMSO, E2, ICI, and E2 plus ICI or tamoxifen and E2 plus tamoxifen. Luciferase activity was determined as described in Materials and Methods. Significant ($p < 0.05$) induction is indicated by an asterisk (*), inhibition is indicated by double asterisks (**) and the results are expressed as means + SE for three replicate determinations for each treatment group.

E2F-1 promoter that determine cell context-dependent differences in their hormone-responsiveness.

Dominant negative NFYA (4YA13m29) interacts with NFYB but the resulting complex does not bind CCAAT sites (397, 398). In MCF-7 cells transfected with pE2F-1j, dominant negative NFYA inhibited E2-induced transactivation, whereas E2-inducibility is decreased but not lost in ZR-75 cells (Fig. 25). Overexpression of 4YA13m29 in ZR-75 cells also blocked activation of a construct containing the CCAAT sites (pE2F-1jm1) but not the GC-rich sites (pE2F-1k) (Fig. 26) confirming the hormone-inducibility of the CCAAT motifs in this cell line. Transfection with NFYA or Δ 4YA13 (a long form of NFYA) did not affect hormone-responsiveness. Interactions between NFYA and ER α have previously been reported on the human coagulation factor XII promoter where NFYA inhibits ER α -mediated transactivation from motifs which contain an overlapping CCAAT/nonconsensus ERE site (376). NFYA also inhibits hormone-induced transactivation in NIH3T3 and human HepG2 cells transfected with ER α and a construct containing a consensus ERE promoter (376). In contrast, our results in ZR-75 cells show that NFYA does not inhibit ER α /Sp1 action in cells transfected with a GC-rich construct (pE2F-1k; Fig. 24) nor does NFYA inhibit hormone-induced transactivation from an ERE promoter in ZR-75 cells (Fig. 27). Thus, inhibitory NFYA-ER α interactions are also cell context-dependent.

The unique hormone-dependent activation of constructs containing CCAAT motifs that bind NFYA was not accompanied by increased binding to these sites as determined in gel mobility shift assays (Fig. 28). A recent report showed that cAMP induced transactivation of human tissue inhibitor of metalloproteinases-2 through activation of NFYA bound to a CCAAT site (384). E2 activates the cAMP/PKA pathway in several cell lines including breast cancer cells (175, 186, 385-387). Studies using the *bcl-2* and cyclin D1 genes revealed that E2 activates the cAMP/PKA pathway in MCF-7 and ZR-75 cells (Fig. 54) (175, 177). Therefore we further investigated the role of this non-genomic pathway in E2-dependent activation of an E2F-1-derived construct containing CCAAT motifs (pE2F-1_{j_{m1}}) in ZR-75 cells. The results in Figure 30 show that hormonal activation of pE2F-1_{j_{m1}} is inhibited by the adenyl cyclase inhibitor SQ22536; E2 (in the absence of cotransfected ER α), 8-bromo-cAMP and constitutively active PKA also activate pE2F-1_{j_{m1}} (Fig. 29), and dominant negative NFYA inhibits E2 and PKA induction of the same construct (Fig. 32). Moreover, E2-dependent activation of the GAL4-NFYA fusion protein is also inhibited by SQ22536 in ZR-75 cells, and this was consistent with comparable inhibition of pE2F-1_{j_{m1}}. This represents a novel non-genomic pathway for activation of NFYA by E2 and is consistent with reports in other cell lines showing cAMP/PKA-dependent activation of NFYA as illustrated in Fig. 54 (384). The precise mechanisms of extranuclear ER α interactions with adenyl cyclase

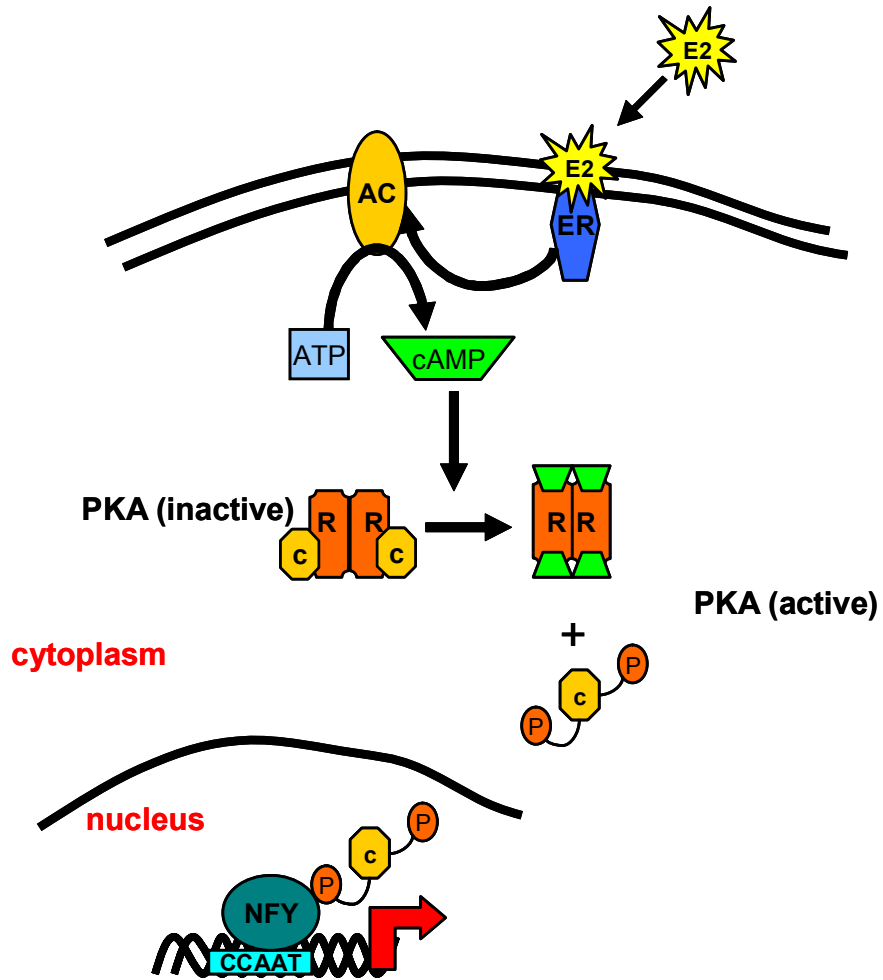


Fig. 54. cAMP/PKA activation of NFY proteins in ZR-75 cells. E2 treatment activates adenyl cyclase, an enzyme that catalyzes the conversion of ATP to cAMP. Production of cAMP causes the dissociation of inactive PKA into its regulatory and active catalytic subunits. The catalytic subunits of PKA translocate into the nucleus and phosphorylate transcription factors such as NFY. AC-Adenyl cyclase, R-regulatory subunit of PKA, C-catalytic subunit of PKA, P-phosphorylation.

are unknown. However, activation of this enzyme results in enhanced cAMP production and release of the active catalytic form of PKA. The precise site(s) of phosphorylation on NFY are unknown. However, NFYB, a regulatory subunit of the NFY complex, was found to be the target site for cAMP signaling on the ferritin promoter (399).

Non-genomic pathways activated by E2 have been characterized in multiple cell lines including breast cancer cells (189, 190, 205, 400, 401). The mechanisms associated with these pathways are complex and may be dependent on several factors including cell context and ER-subtype (192, 402). Results of this study clearly demonstrate that cell context (MCF-7 vs. ZR-75) is an important factor in hormonal regulation of E2F-1 gene expression, and in ZR-75 cells, a combination of both genomic (ER α /Sp1) and non-genomic (cAMP/PKA) signaling is required. Interestingly, a combination of these pathways has also been reported for induction of c-fos, cyclin D1 and bcl-2 in MCF-7 or ZR-75 cells (173, 215, 259). These gene promoters all contain E2-responsive GC-rich motifs as well as cAMP response elements (bcl-2 and cyclin D1) (Fig. 55) or a serum response element (c-fos) (Fig. 56) activated through mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) (215, 259, 403). Since inhibitors of MAPK and PI3-K pathways block E2-induced proliferation of MCF-7 cells (215, 259, 403), the identification of

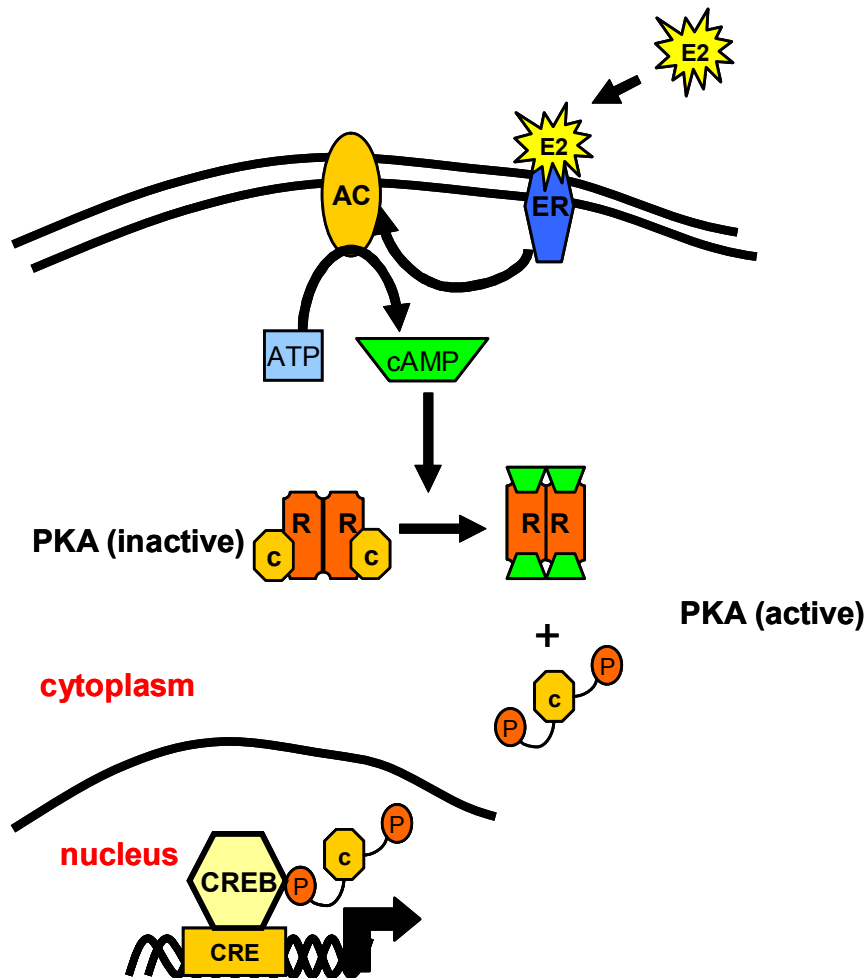


Fig. 55. Bcl-2 (MCF-7) and cyclin D1 (ZR-75) non-genomic pathways. Bcl-2 and cyclin D1 promoters contain CREs in their promoter regions that are bound by CREB. Upon E2 treatment, adenyl cyclase is activated and catalyzes the conversion of ATP to cAMP. cAMP activity induces the dissociation of inactive PKA into its regulatory and active catalytic subunits. The catalytic subunits translocate into the nucleus where they activate CREB by phosphorylation. CRE – cAMP response element, CREB – CRE binding protein (175, 177).

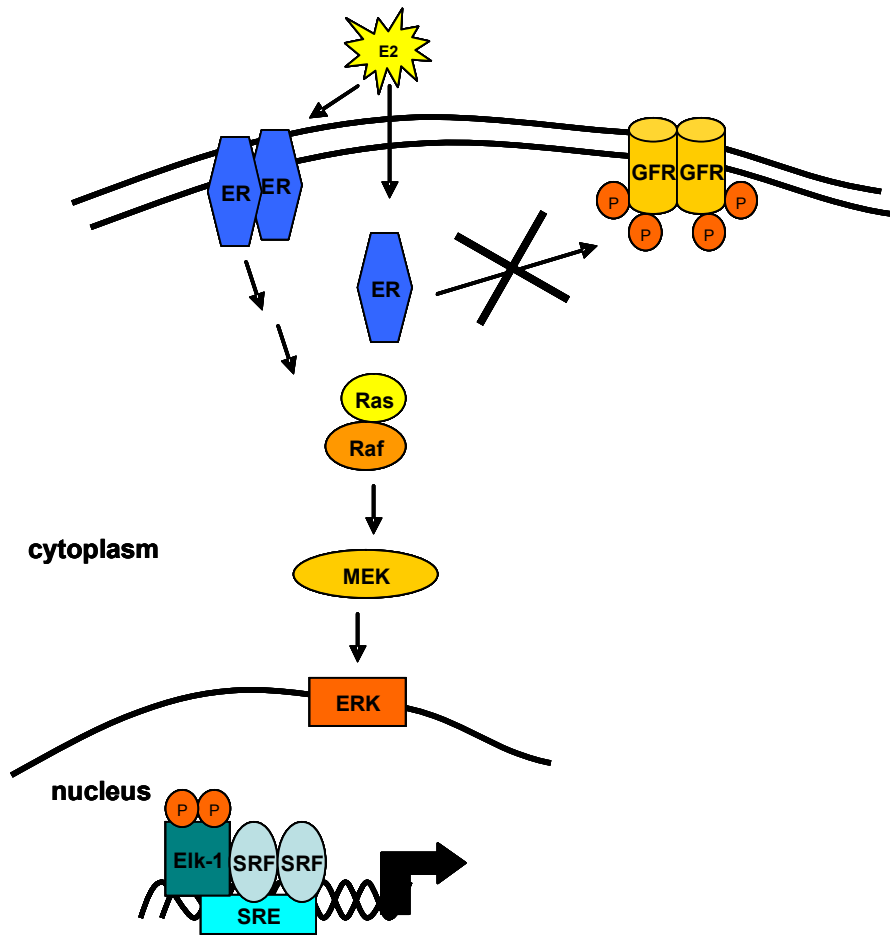


Fig. 56. Estrogen and growth factor activation of Elk-1 and a serum response element (SRE) in the *c-fos* gene promoter. The *c-fos* gene promoter contains an SRE that is bound by Elk-1 and SRF. E2 treatment activates the MAPK pathway that eventually phosphorylates Elk-1 thereby activating transcription. GFR – growth factor receptor, SRF – serum response factor.

downstream E2-responsive gene targets such as E2F-1, cyclin D1, c-fos and bcl-2 is consistent with the contributions of non-genomic pathways of estrogen action. Current studies are investigating the mechanisms of non-classical genomic and non-genomic pathways on growth regulatory genes in breast cancer cells and determining cellular factors that influence cell context-dependent mechanistic differences, even among ER-positive breast cancer cell lines. The possibilities include the recruitment of corepressors that may inhibit transactivation in one cell line whereas coactivators may be recruited to induce transactivation in another cell line.

4.2 Inhibition of E2-induced Expression of E2F-1 by TCDD

E2F-1 and related proteins play an important role in cell proliferation and phosphorylation-dependent dissociation of the E2F-1-Rb complex results in release of E2F-1 and subsequent activation of E2F-1 –dependent genes (352-354, 404). The oncogenic potential of E2F-1 is associated with overexpression of E2F-1-regulated genes and their enhancement of cell growth (Fig. 57) (352-354, 394-396, 404). In contrast, E2F-1 also exhibits tumor suppressor activity and E2F-1^{-/-}/E2F-1^{+/+} mice show early development of hyperplastic and neoplastic responses (390, 391). Overexpression of E2F-1 in some cancer cell lines results in activation of cell death pathways suggesting a possible mechanism for E2F-1-dependent tumor growth inhibition (Fig. 57) (405).

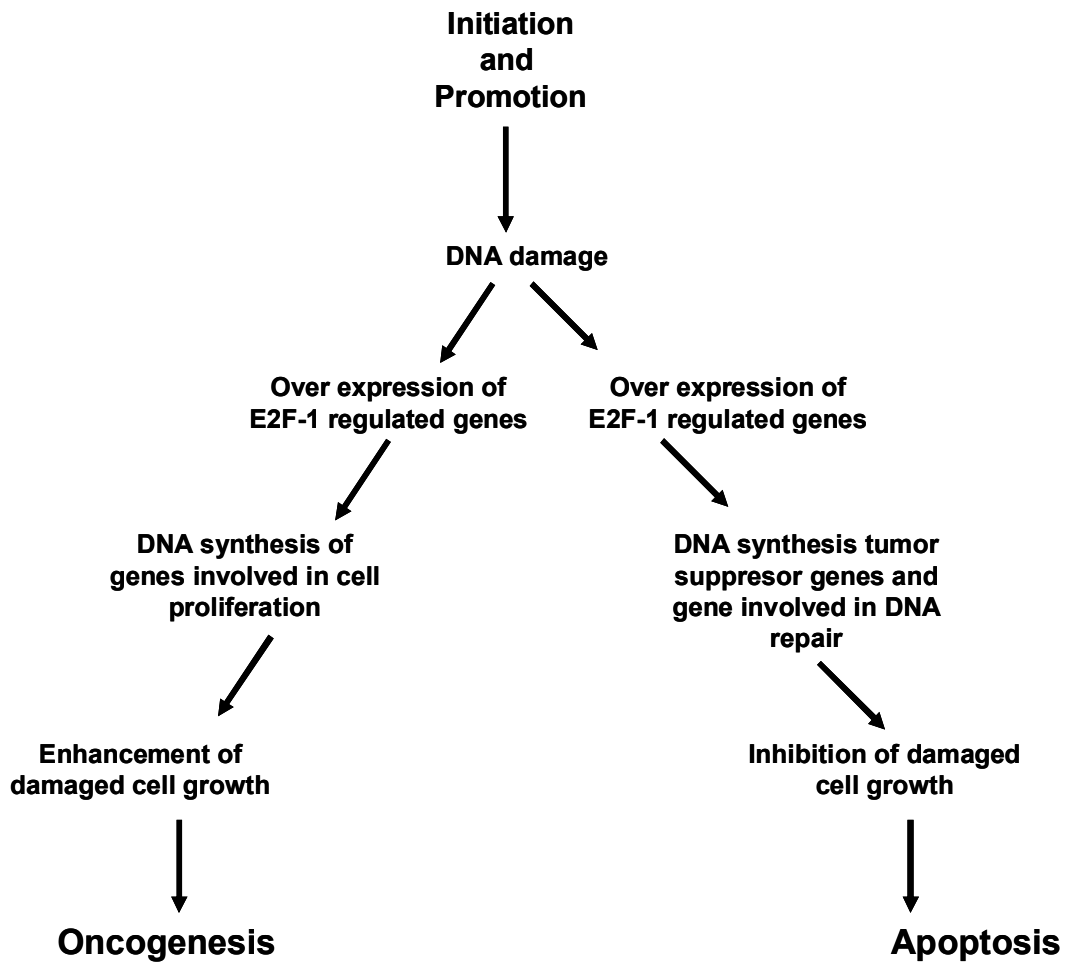


Fig. 57. E2F-1 as an oncogene and a tumor suppressor. E2F-1 has the ability to be an oncogene as well as a tumor suppressor. When DNA is damaged, there is an overexpression of E2F-1 regulated genes that are either involved in cell proliferation or apoptosis and DNA repair. If genes involved in cell proliferation are overexpressed, E2F-1 promotes oncogenesis and if apoptosis genes are overexpressed, E2F-1 activates the cell death pathways.

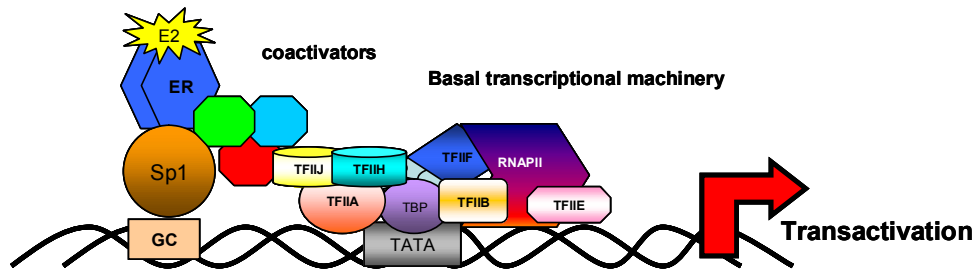
E2-dependent regulation of E2F-1 in MCF-7 and ZR-75 cells is primarily associated with increased proliferation. Previous studies showed that E2-responsiveness in both cell lines was associated with the proximal GC-rich (-169 to -11) and CCAAT (-111 to -54) elements; however, as indicated in the previous section there were significant differences in mechanisms of hormone-dependent activation (342, 343). ER α /Sp1-dependent activation through interactions with the GC-rich elements was observed in both cell lines; however, in MCF-7 cells E2-induced transactivation required cooperative interactions with downstream NF-YA transcription factors whereas in ZR-75 cells both GC-rich and CCAAT sites were independently activated by E2. Estrogen-responsive genes and tumors are inhibited by antiestrogens such as tamoxifen and ICI 182,780 and these same compounds are also used for treatment of breast cancer in women. Other compounds such as retinoids (406, 407), PPAR γ (408) and vitamin D receptor agonists (409-411) also inhibit mammary tumor growth, and research in our laboratory has observed inhibitory AhR-ER α crosstalk which includes inhibition of mammary tumor growth (272, 273, 412). The mechanisms of these interactions between the AhR and ER α signaling pathways are complex and involve interactions of the AhR with specific promoter regions (iDREs) of some E2-responsive genes (262), proteasome-dependent downregulation of ER α (368), and induction of “inhibitory” factors such as HES-1 (388, 389). The mechanisms of inhibitory AhR-ER α cross-talk using the E2F-1 gene as a model

were investigated in MCF-7 and ZR-75 breast cancer cells to take advantage of their subtle differences in hormonal regulation of E2F-1.

TCDD inhibited E2-induced E2F-1 mRNA and/or protein levels in MCF-7 (Fig. 34) and ZR-75 (Figs. 37 and 38) cells, and similar interactions were observed in cells transfected with pE2F-1 constructs (Figs. 35,36,39 and 40). Previous studies have demonstrated that direct interactions of the AhR complex with specific iDREs can inhibit E2-induced expression of cathepsin D, c-fos, pS2 and heat shock protein 27, and this is associated with AhR squelching or disrupting of a functional ER α or ER α -protein complex (32-37). The proximal region of the E2F-1 gene promoter does not contain a iDRE motif (GCGTG), and the decreased transactivation in both cell lines transfected with various E2F-1 constructs must be due to other mechanisms. It was not possible to determine the importance of limiting levels of ER α expressed in cells after treatment with TCDD plus E2 since the proteasome inhibitor MG132 alone prevented hormone-dependent activation of E2F-1 (Figs. 41 and 42). It is possible that levels of ER α in these cells after treatment with TCDD plus E2 may contribute to the antiestrogenic effects of TCDD as previously reported (135, 368). We also investigated these responses in SL-2 cells which do not express ER α , Sp1, or AhR/Arnt. In cells transfected with pE2F-1h, ER α and Sp1 expression plasmids (Fig. 44A) E2 significantly induced transactivation. These data are consistent with a previous report showing hormone-induced transactivation in SL-2 cells

transfected with ER α and Sp1 expression plasmids and constructs containing GC-rich promoter inserts from the DNA polymerase α , thymidylate synthase, TGF α , and bcl-2 genes (10,53-55). In SL-2 cells transfected with pE2F-1h and AhR/Arnt expression plasmids, TCDD did not affect luciferase activity (Figs. 43C and 44B). However, in cells cotransfected with ER α (250 ng)/Sp1(100 ng) (E2-responsive, Fig. 44A) and the lowest amount of AhR/Arnt (50 ng), TCDD significantly inhibited E2-induced luciferase activity (Fig. 45) and the results were comparable to inhibitory responses observed in MCF-7 (Fig. 36A) and ZR-75 (Figure 39A) cells transfected with the same constructs. Increasing amounts of the AhR complex (unliganded) (100-500 ng) slightly decreased hormone-induced transactivation due to parallel increase in basal (DMSO) activity (250 and 500 ng) (Fig. 44A). Luciferase activity in SL-2 cells treated with TCDD plus E2 was significantly lower than activity in cells treated with E2 alone and transfected with 50, 100, 250, or 500 ng AhR/Arnt. The results suggest that the liganded AhR complex represses ER α /Sp1 and this is consistent with previous reports showing that AhR/Arnt interacts directly with ER α and Sp1 proteins (339, 341). The model shown in Fig. 58 shows that AhR/Arnt acts as a ligand-induced corepressor that interacts with ER α /Sp1 and prevents subsequent recruitment of coactivators and basal transcriptional machinery.

(A)



(B)

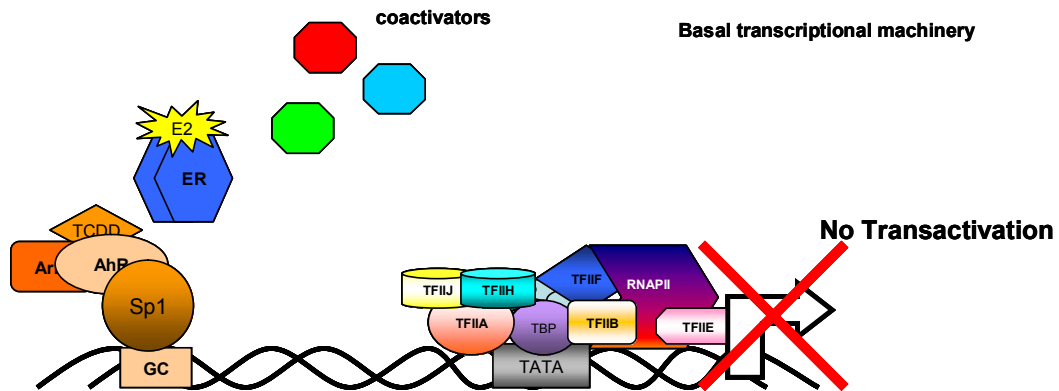


Fig. 58. AhR/Arnt-mediated inhibition of ERα/Sp1 transactivation. (A) ERα/Sp1-mediated transactivation is a result of the recruitment of coactivators that interact with the ERα/Sp1 complex and the basal transcription machinery. (B) AhR/Arnt interaction with Sp1 inhibits ERα-mediated responses by preventing ERα from interacting with Sp1 and thereby not allowing interaction between the coactivators and the basal transcription machinery (233, 413).

The studies in MCF-7, ZR-75 and SL-2 cells demonstrate that AhR agonists inhibit genomic ER α /Sp1-mediated transactivation. However, in ZR-75 cells TCDD also inhibits E2-dependent activation of pE2F-1d and pE2F-1j. These constructs contain two hormone responsive CCAAT sites, which are activated through non-genomic cAMP/PKA pathways (343). It has been recently reported that TCDD induces the transcriptional inhibitor HES-1 in T47D cells (388) and the E2F-1 promoter contains a HES-1 response element at -41 to -36 (CACGAG) (389) and the involvement of HES-1 could explain the inhibitory effects of TCDD on constructs containing both CCAAT and HES-1 sites. However TCDD inhibits E2-dependent transactivation in cells transfected with pE2F-1d and pE2F1j. The latter construct does not contain that HES-1 response element suggesting the HES-1 activation is not involved in the inhibitory response induced by TCDD. Since TCDD also inhibits E2-dependent phosphorylation of PKA (Fig. 47), we further investigated E2/TCDD interactions on regulation of adenylyl cyclase activity (Table 4). These results demonstrate that induction of cAMP by E2 was inhibited by both TCDD and the specific adenylyl cyclase inhibitor SQ22536. Previous studies in human granulosa cells showed that TCDD also decreased PKA activity (414) and in this study we have also observed inhibition of PKA activity by TCDD in cells treated with 8-bromo-cAMP or transfected with a constitutively-active PKA expression plasmid (Fig. 48). Thus, TCDD can inhibit both adenylyl cyclase activity and PKA-dependent

phosphorylation, and this results in the failure to activate NFY proteins in the proximal regions (-122 to -54) of the E2F-1 promoter (Fig. 59). These results demonstrate that TCDD inhibits E2-dependent activation of both non-genomic and genomic pathways of ER activation associated with the E2F-1 gene/gene promoter. The effects are similar to those observed for some antiestrogens such as ICI 182,780 which also inhibits genomic and non-genomic pathways of E2 action and like TCDD also induces proteasome-dependent degradation of ER α (190, 415). Ohtake and coworkers (340) reported that Ah receptor agonists such as TCDD and 3-methylcholanthrene exhibited estrogenic activity through AhR-ER α interactions where ER α acts as a DNA bound transcription factor and the liganded AhR exhibits coactivator activity. In contrast, results of this study suggest that the ligand bound AhR complex corepresses ER α /Sp1. This type of interaction has also not been observed in other studies *in vitro* and *in vivo* (135, 262, 291, 292, 296, 331, 332, 334, 366-368) and is currently being reexamined in this laboratory. Current studies are also investigating mechanisms of AhR-mediated inhibition of other E2-responsive genes and development of selective AhR modulators for treatment of breast cancer.

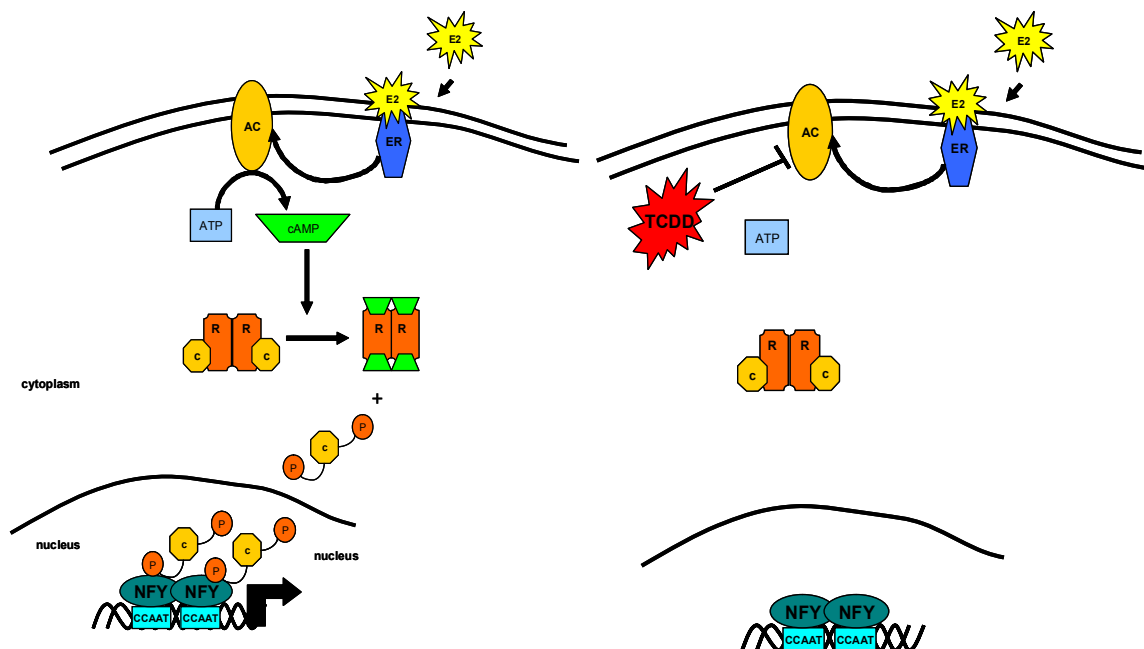


Fig. 59. Inhibition of E2-dependent activation of cAMP/PKA by TCDD and the E2F-1 gene promoter in ZR-75 cells. E2 induces cAMP/PKA activation of the CCAAT motifs on the E2F-1 promoter. TCDD treatment inhibits adenyl cyclase activation and subsequently prevents PKA and NFY phosphorylation thereby inhibiting E2-mediated responses.

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VITA

PERSONAL

Sharon Khethiwe Ngwenya
1778 Harmony Hills Dr.
Lithonia, Georgia 30058

EDUCATION

2005 Biochemistry, Ph.D
Texas A&M University
College Station, Texas

1998 Biochemistry, B.S
Oakwood College
Huntsville, Alabama

PUBLICATIONS

- 2004 Qin, C, Samudio, I, Ngwenya, S, and Safe, S Estrogen-dependent regulation of ornithine decarboxylase in breast cancer cells through activation of nongenomic cAMP-dependent pathways. *Mol Carcinog*, 2004; *40*(3): 160-170.
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